Imaging nociceptive signaling in peripheral CGRP terminal fibres

A Thesis Submitted to the College of

Graduate Studies and Research

In Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

In the Department of Physiology

University of Saskatchewan

Saskatoon

By

Landon D. Baillie

Permission to Use

In presenting this thesis/dissertation in partial fulfilment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis/dissertation in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis/dissertation work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis/dissertation or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Physiology
University of Saskatchewan
Saskatoon, Saskatchewan S7N5E5

ABSTRACT

In this dissertation I introduce a simple experimental approach for studying afferent pain fibre physiology. I developed an *en bloc* dural-skull preparation that pairs electrophysiological stimulations, pharmacological manipulations, and the UV photolysis of caged compounds in and around selectively identified individual C-fibre nociceptors with microfluorometric imaging of Ca^{2+} responses. This allows the observation of physiological functioning in individual nociceptive fibre free nerve endings. I show high-resolution functional imaging of single action potential-evoked fluorescent transients, as well as sub- and supra-threshold calcium signaling events within individual nociceptive fibre terminations.

Utilizing the dural-skull preparation I was able to identify a peripheral mechanism of action in the terminals of CGRP nociceptive fibres for an effective migraine therapeutic, the selective 5-HT1 receptor agonist, sumatriptan. I found sumatriptan to cause an approximately 40% reduction in the amplitude of action potential-evoked Ca^{2+} transients in the peripheral terminals of CGRP nociceptive fibres that was mediated selectively through the inhibition of N-type Ca^{2+} channels. Observations from this study support a peripheral site of action for sumatriptan in inhibiting the activity of dural pain fibres and adds to our understanding of the mechanisms that underlie the clinical effectiveness of 5-HT1 receptor agonists such as sumatriptan.

While μ-opioid receptor agonists remain the most powerful analgesics for the treatment of severe pain, their mechanism of action in peripheral primary afferent pain fibres remain to be established. Further exploiting the dural-skull preparation I found activation of μ-opioid receptors in individual CGRP terminals had a dual modulatory effect; inhibition of N-type Ca^{2+} channel signaling and a frequency dependent, BK_{Ca} channel-mediated, suppression of action potential firing. These results establish possible anti-nociceptive mechanisms of μ-opioid receptor activation in the peripheral terminals of CGRP nociceptive fibres and identify new pathways to target for peripherally mediated analgesia.

The development and subsequent testing of the dural-skull preparation in this dissertation displays its utility and opens up a new window for studying nociceptive fibre physiology and pathophysiology.

ACKNOWLEDGEMENTS

Firstly I would like to thank my supervisor, Dr. Sean Mulligan. Sean supplied me with unlimited resources, opportunity and most importantly, his time, all of which helped mould me into the scientist I am today. The daily scientific banter in his office was an invaluable resource during my studies. Aside from being a most excellent mentor, Sean has become one of my best friends, that of which I am most appreciative. I would also like to thank Sean's wife, Kelly, for her friendship and for putting up with Sean and I's incessant emailing back and forth at all hours.

Thank you to my committee members, Dr. Thomas Fisher, Dr. Valerie Verge, Dr. Veronica Campanucci, and Dr. Nigel West for providing helpful criticisms and comments along my travels and thank you to Dr. Andrew Ahn and Dr. Helmut Schmidhammer for supplying valuable reagents.

I would like to thank my best friend and partner Laura Pugh. Your love, encouragement and tolerance are one of the greatest resources I could have asked for during the course of my PhD, I love you.

And finally thank you to my friends and family for making me the man I am today.

TABLE OF CONTENTS

PAGE

LIST OF FIGURES

Tables

LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
5-HT_{1B}	5-hydroxytryptamine (serotonin) receptor 1B
5-HT_{1D}	5-hydroxytryptamine (serotonin) receptor 1D
ACC	Anterior cingulate cortex
aga-IVA	Agatoxin-IVA
AMPA	α-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid
AP	Action potential
Aδ	A-delta
BK_{Ca}	Big conductance calcium activated potassium channel
BSA	Bovine serum albumin
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
CCD	Charge coupled device
CeA	Central nucleus of the amygdala
CGRP	Calcitonin gene-related peptide
Cl^-	Chloride
CLR	Calcitonin-like receptor
CNS	Central nervous system
Cs^+	Cesium
CSD	Cortical spreading depression
ctx-GVIA	Conotoxin GVIA
DRG	Dorsal root ganglion

EGFP	Enhanced green fluorescent protein
Gabaergic	γ-aminobutyric acid containing
IB4	Isoletctin B4
IC	Insular cortex
K^+	Potassium
KCl	Potassium chloride
LC	Locus ceruleus
LDCV	Large dense core vesicles
$MgCl_2$	Magnesium chloride
MOR	mu-opioid receptor
Na^+	Sodium
NaCl	Sodium chloride
NaH_2PO_4	Monosodium phosphate
$NaHCO_3$	Sodium bicarbonate
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NP-EGTA	o-Nitrophenyl-ethylene glycol tetraacetic acid
OGB-1 AM	Oregon green bapta-1 AM
P2X3	Purinergic receptor P2X, ligand-gated ion channel, 3
PAG	Periaquaductal grey
PBS	Phosphate buffered saline
PFC	Prefronta cortex

PMT	Photomultiplier tube
RAMP1	Receptor activity modifying protein type 1
RCP	Receptor component protein
Ret	Ret family of tyrosine kinase receptors
Runx1	Runt-related transcription factor 1
RVM	Rostral ventromedial medulla
S1	Somatosensory cortex
TRG	Trigeminal root ganglion
Trka	Tropomyosin receptor kinase A
TRP	Transient receptor potential
TRPA1	Transient receptor potential cation channel, subfamily A, member 1
TRPM8	Transient receptor potential cation channel, subfamily M, member 8
TRPV1	Transient receptor potential cation channel, subfamily V, member 1
TRPV2	Transient receptor potential cation channel, subfamily V, member 2
TRPV3	Transient receptor potential cation channel, subfamily V, member 3
TRPV4	Transient receptor potential cation channel, subfamily V, member 4
TTX	Tetrodotoxin
UV	Ultraviolet
μ-Opioid	Mu-opioid

CHAPTER 1

INTRODUCTION

1.1 Nociceptive signaling

Pain is described as the subjective, unpleasant sensory and emotional experiences associated with actual or potential tissue damage (Kandel, 2013). The ability to sense noxious stimuli is essential to the survival and well being of many organisms and leads to appropriate protective behaviours to avoid or retract from conditions that may be life threatening (Basbaum et al., 2009).

The sensation of pain commonly begins with nociception, the process by which intense thermal, mechanical or chemical stimuli is sensed by a subset of peripheral sensory nerve fibres known as nociceptors (Gold and Gebhart, 2010) . Nociceptors are pseudounipolar neurons whose cell bodies reside in the dorsal root ganglion or trigeminal ganglion. From the cell body both peripheral and central terminals emanate from a common axonal stalk, one extending out to the periphery, the other entering into the dorsal horn of the spinal cord or brainstem (Figure1.1B) (Basbaum et al., 2009).

The peripheral terminals of nociceptors contain a variety of transducing channels that convert mechanical, chemical and thermal stimuli into electrical activity and thus action potentials in the nociceptors that travel back to the central nervous system (CNS) (Figure 1.1A) (Woolf and Ma, 2007).

Nociceptive signals are transmitted at a central synapse in the spinal cord or hind brain through the release of a variety of excitatory neurotransmitters such as glutamate, calcitonin gene-related peptide (CGRP) or substance P that have the potential to excite second order

nociceptive projection neurons (Grace et al., 2014). The majority of primary-secondary nociceptive synapses occur within the superficial laminae of the spinal dorsal horn (Kuner, 2010). Nociceptive signaling can be modulated at the central synapse in the spinal cord via activation of γ-aminobutyric acid containing (gabaergic) and glycinergic inhibitory interneurons (Takazawa and MacDermott, 2010).

The majority of secondary projection neurons travel up the spinothalamic tract of the spinal cord and project to supra-spinal sites, primarily the thalamus, where this nociceptive information is then relayed to numerous cortical and subcortical regions via third order neurons allowing for encoding and perception of the painful experience (Grace et al., 2014). Aside from the spinothalamic tract, secondary projection neurons also ascend to the reticular formation and thalamus via the spinoretricular tract and the periaqueductal gray and mesencephalic reticular formation via the spinomesencephalic tract (Kandel, 2013).

Primary-secondary nociceptive signaling in the spinal cord can be modulated by activation of descending pathways originating primarily in the periaqueductal gray of the midbrain (Heinricher et al., 2009). Neurons in the periaqueductal gray receiving nociceptive information from the spinomesencephalic tract project to and activate noradrenergic and serotonergic neurons in the locus ceruleus and nucleus raphe magnus of the brainstem (Kandel, 2013). These neurons from the locus ceruleus and nucleus raphe magnus descend to the dorsal horn of the spinal cord where they synapse on and activate endogenous opioid containing interneurons (Heinricher et al., 2009). The release of endogenous opioids results in activation of opioid receptors on primary nociceptors and secondary projection neurons in the spinal cord. Opioid receptor activation in the primary nociceptors leads to the inhibition of a variety of Ca^{2+} channels and a subsequent decrease in synaptic release and in the secondary projection neurons

activates K^+ channels causing hyperpolarization, leading to a decrease in excitation and/or propagation of action potentials (Stein C and Zollner C, 2009).

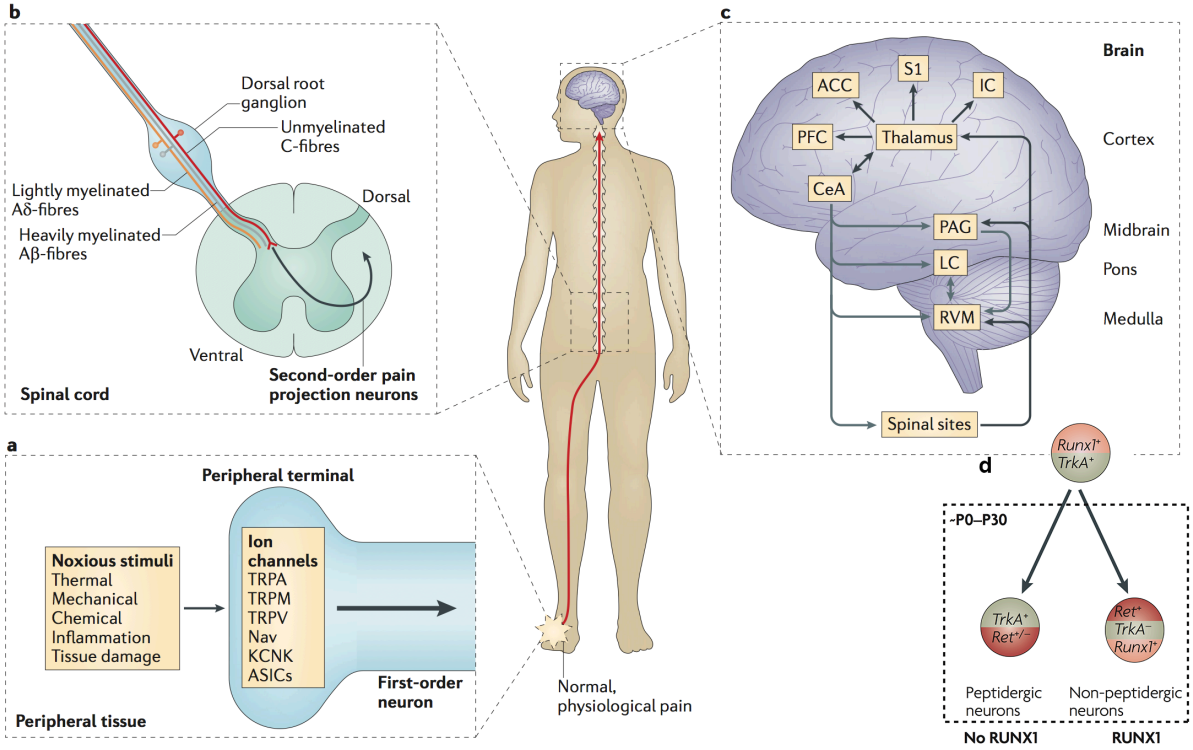

Figure 1.1. Overview of nociceptive signaling pathways.
A) Highlights the transduction channels in the peripheral terminals of nociceptors responsible for sensing noxious stimuli. **B**) Nociceptors from the periphery travel back to the CNS and enter the dorsal horn of the spinal cord and synapse on secondary projection neurons or interneurons. The cell bodies of nociceptors are located outside the spinal cord in the dorsal root ganglion or outside the brainstem in the trigeminal ganglion. **C**) Highlights the areas involved in the conscious sensation of pain and the areas of the brain that have descending outputs to the spinal cord to modulate incoming nociceptive signals. **D**) The developmental pathways of the two main subpopulations of C-nociceptors; peptidergic and non-peptidergic. PFC; prefrontal cortex, CeA; central nucleus of the amygdala, ACC; anterior cingulate cortex, S1; somatosensory cortex, IC; insular cortex, LC; locus ceruleus, PAG; periaqueductal gray, RVM; rostral ventromedial medulla. Adapted from (Grace et al., 2014; Marmigere and Ernfors, 2007)

1.2 Primary afferent nociceptors

There are two main classes of nociceptors, unmyelinated slow conducting C nociceptors (C fibres) and lightly myelinated more rapidly conducting Aδ nociceptors (Aδ fibres). Aδ nociceptors usually have medium to large sized cell bodies (>45 μm in diameter) and are capable of sensing noxious mechanical stimuli and a subset of them are also capable of transmitting noxious thermal stimuli; whereas the majority of C nociceptors have small to medium sized cell bodies (<45 μm in diameter) and are polymodal making them capable of sensing noxious thermal, mechanical and chemical stimuli (Julius and Basbaum, 2001). An additional class of C nociceptors exist known as silent nociceptors, which are polymodal nociceptors that only become responsive to noxious thermal and mechanical stimuli after sensitization from inflammatory mediators but in the absence of inflammatory mediators remain unresponsive (Dubin and Patapoutian, 2010).

1.2.1 Subpopulations of C fibres

C-fibres constitute the majority of nociceptors found throughout the body and are commonly broken down into two subpopulations, on the basis of cell content of receptor expression, peptidergic and non-peptidergic. Peptidergic nociceptors are characterized by the presence of one or both of the neuropeptides calcitonin gene-related peptide (CGRP) and substance P and are further defined by the presence of the nerve growth factor receptor, tropomyosin receptor kinase A (TrkA) (Gold and Gebhart, 2010). Non-peptidergic nociceptors are characterized by the expression of Ret, the tyrosine kinase receptor that is the signaling component for the neurotrophic factors of the glial-derived family (Franck et al., 2011); the presence of purinergic P2X3 receptors and the ability to bind and be labeled by an isolectin known as IB4 (Julius and Basbaum, 2001). Peptidergic and non-peptidergic subpopulations are

often further sub-divided based on their primary stimulus response or the absence or presence of certain ion channels such as transient receptor potential V1 (TRPV1) or tetrodotoxin (TTX)-resistant Na^+ channels (Handwerker, 2010).

1.2.2 Development

The two populations of C nociceptors differentiate during peri/postnatal development and depend on genetic mechanisms (Abdel Samad et al., 2010). Runt-related transcription factor 1 (Runx1), plays a pivotal role in determining the two subpopulations of C fibres. Initially all nociceptors express TrkA and Runx1 during development and require nerve growth factor (NGF) for survival during embryonic life (Snider and McMahon, 1998). In non-peptidergic nociceptors Runx1 expression remains, TrkA expression is switched off and Ret expression on, whereas in the peptidergic nociceptors Runx1 expression switches off and TrkA expression is retained and occasionally Ret may also be expressed (Abdel Samad et al., 2010) (Figure 1.1D).

1.2.3 Transduction

The transduction of mechanical, chemical and thermal stimuli in nociceptors is initiated by the generation of depolarizing generator potentials created through the activation of unique protein molecules or receptors found in the peripheral terminals of C-fibres (Figure 1.2A+B, Table 1) (Gold and Gebhart, 2010). When these receptors encounter the appropriate specific stimulus (e.g., high heat, extreme cold, chemicals, or excessive pressure) of sufficient intensity, the receptor molecule undergoes a conformational change that transduces the noxious signal into an electrical current (generator potential) by triggering the opening of depolarizing cationic ion channels, sodium/calcium (Na^+/Ca^{2+}) or the closing of outward potassium channels (K^+) (Babos et al., 2013). If the generator potential is of sufficient magnitude or spread, nearby voltage gated Na^+ channels become activated, initiating an action potential in the nociceptor.

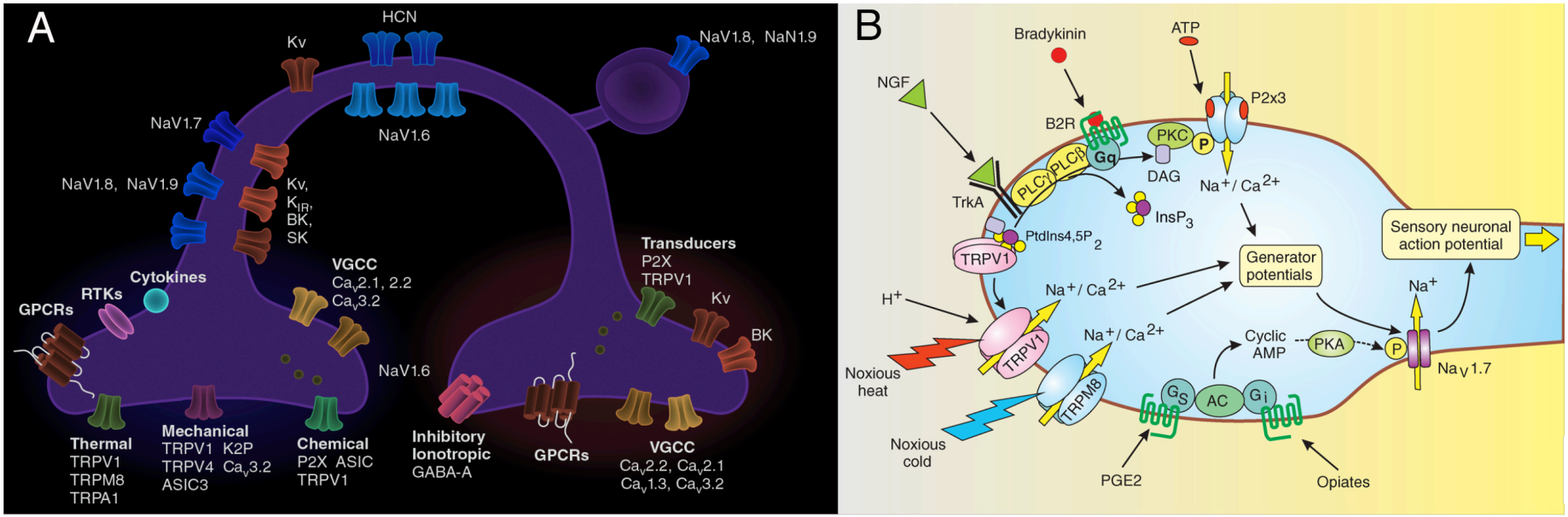

Figure 1.2. Transduction in peripheral nociceptive terminals.
A) Highlights the thermal, mechanical and chemical transduction channels that have been found in the peripheral terminals of nociceptors. **B**) Noxious transduction channels create small depolarizations known as generator potentials that activate low threshold voltage gated Na^+ channels and lead to action potentials that travel back to the CNS. Adapted from (Gold and Gebhart, 2010).

Table 1. Primary afferent transducers

Mechanical	Thermal	Chemical
ASIC1	TRAAK/TREK-1	ASIC 1-4
ASIC2	Nav1.8	TRAAK/TREK
ASIC3	TRPA1	TRPV1
Cav3.2	TRPM8	TRPA1
TRPV1	TRPV3	TRPM8
TRPV4	TRPV4	P2X1-6
TRPA1	TRPV1	5-HT3
TRAAK	TRPV2	nAch
TREK1/2		GluR-1, NR1-2
P2X3		GABA

Table 1. List of mechanical, thermal and chemical transducing channels found in primary afferent nociceptors. Adapted from Gold and Gebhart 2010.

1.2.4 Transduction channels

A wide variety of ion channels have now been discovered that can directly or indirectly transduce thermal, mechanical and chemical stimuli (Table 1) (Gold and Gebhart, 2010). The transient receptor potential (TRP) superfamily of channels appears to be of particular importance (Figure 1.2A). The eight subfamilies of TRP channels share a few common features including consisting of six transmembrane segments, a varying degree of sequence homology and permeability to cations but despite their similarities display a diversity of cation selectivity and varying mechanisms of activation (Venkatachalam and Montell, 2007). Since the cloning of the first discovered TRP channel involved in pain, the capsaicin receptor (TRPV1) (Caterina et al., 1997), a wide range of TRP channels have been found to be expressed in nociceptors including TRPV2, TRPV3, TRPV4, TRPM8, and TRPA1 and have been found to transduce a variety of noxious stimuli (Wang and Woolf, 2005).

Of all the TRP channels identified, the initially discovered TRPV1 channel has been the most studied. TRPV1 is found to be highly expressed in nociceptors and is considered to be a polymodal receptor capable of being activated by voltage and noxious thermal and chemical stimuli (Oh, 2006) and has been found to be involved in mechanical hyperalgesia (Honore et al., 2005). Chemical activators of TRPV1 channels include capsaicin, (the main pungent ingredient in hot peppers), and related vanilloids, protons and endocannabinoids such as anandamide (Wang and Woolf, 2005). Being a polymodal receptor TRPV1 is also activated by noxious levels of heat with temperatures >42^{o}C acting directly on the channel gating to open the TRPV1 channel resulting in a nonselective cation current (Cesare and McNaughton, 1996; Reichling and Levine, 1997).

The method by which noxious thermal and chemical stimuli initiate the opening of the TRPV1 channel resulting in a cation influx have recently been determined. Temperatures above 42^{o}C result in the pore undergoing a structural change resulting in the influx of cations (Grandl et al., 2010) whereas activation by vanilloids such as capsaicin is restricted to a small cytosolic segment spanning transmembrane 2-4 of the receptor (Jordt and Julius, 2002) and activation by protons is restricted to an extracellular domain (Jordt et al., 2000).

The TRPV1 channel is permeable to various monovalent cations including Na^+, K^+, cesium (Cs^+) and Ca^{2+} (Oh et al., 1996), but it has an approximate 10-fold preference for Ca^{2+} (Chung et al., 2008). Classically the cation, primarily Ca^{2+}, influx through TRPV1 is believed to cause generator potentials in the nociceptive terminal, depolarizing voltage gated Na^+ channels followed by the generation of action potentials. More recently it has also been proposed that action potential generation by TRPV1 activation could occur via an anion efflux mechanism through the Ca^{2+} activated chloride (Cl^-) channel anoctamin 1 (Takayama et al., 2015).

Similarly to TRPV1, other nociceptive channels in Table 1 transduce noxious stimuli into an electrical current (generator potential) primarily by triggering the opening of depolarizing cationic ion channels (Na^+ and Ca^{2+}) or the closing of outward potassium channels (Babos et al., 2013).

1.2.5 Transmission

Three voltage gated Na^+ channels, $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$, are primarily expressed in C nociceptors and are believed to be responsible for initiating and propagating an action potential in response to a sufficient generator potential (Dib-Hajj et al., 2013). $Na_v1.7$ has been the focus of intense research in the pain field due to the discovery of genetic mutations in this channel that can result in a loss of function in the channel and the inability to sense pain, or a gain-of-function

mutation leading to pain hypersensitivity (Cox et al., 2006; Fertleman et al., 2006). $Na_v1.7$ is found in high density near nociceptive endings where it plays the crucial role of amplifying small subthreshold depolarizations (generator potentials) acting as a threshold channel that regulates nociceptor excitability (Figure. 1.2B) (Dib-Hajj et al., 2013). Nav1.9 plays a similar role to 1.7 by acting as a threshold channel (Huang et al., 2014) whereas Nav1.8 is thought to carry the main Na^+ current of the propagating action potential as it travels back to the CNS (Renganathan et al., 2001).

1.3 Current methods for studying nociception

To study nociceptive signaling, identify novel pain targets, and characterize the analgesic potential of new compounds, a variety of cellular and behavioural experimental methods, primarily in rodents, have been developed. While the methods highlighted below have been vital to the current understanding of nociception and development of analgesic targets, the development of novel methods, particularly those that will allow study of the elusive tiny pain-sensing terminals, will be important in moving pain research forward and allowing progress to continue.

1.3.1 Behavioural

One of the largest obstacles in studying nociception in animals is the absence of verbal communication, which limits experimenters to only the study of physical signs of pain by observing "pain-like" behaviours (Xie, 2011). The study of pain-like behaviours most commonly uses tests of simple phasic pain in animals and relies on escape behaviour/withdrawal reflexes or vocalization as an index of pain (Boyce-Rustay et al., 2010).

The behavioural tests of nociception are commonly split based on stimulus into three categories: tests using thermal stimuli, tests using mechanical stimuli, and finally tests using chemical stimuli.

1.3.1.1 Thermal:

The tail flick test was first described by (D'amour and Smith, 1941), and involves restraining a rodent, focusing a heat source from a radiant lamp on the rodents tail and measuring the time from the onset of the stimulus to when the animal feels discomfort and reacts by withdrawing its tail from the heat with a brief vigorous movement (tail flick) (Le Bars et al., 2001). A modified version of the tail flick was later developed that involves immersing the distal

half of the tail into a heated water bath and again measuring the time latency for a tail flick or a complete body movement (Janssen et al., 1963). Note that this method can also be used to test reactivity to cold by using a chilled water bath (Xie, 2011). Since such a large portion of the tail is being exposed to the heat source during the bath version, time latencies are often shorter than the focused light method.

The paw withdrawal test, first developed by (Hargreaves et al., 1988), aimed to behaviourally study hyperalgesia resulting from inflammation. This test involves taking a freely moving rodent on a glass surface and placing a focused infrared source under the plantar surface of a hindpaw when the rodent is not moving. A start button turns on the light source as well as a timer and when the paw is actively removed from the light source it ceases, as does the timer and the time latency from heat onset to withdrawal is recorded.

The hot plate test was first introduced by (Woolfe and Macdonald, 1944) and involves placing a rodent in a open ended acrylic cylinder with a metallic plate floor that is heated by a thermode or boiling liquid to 65^{o}C. At the onset of placement a timer is started and then stopped once the rodent responds with a hind paw lick, flick or jump. A similar device was developed that uses a cooled metallic floor to test behavioural responses to noxious cold (Bennett and Xie, 1988).

1.3.1.2 Mechanical:

Similar to thermal nociceptive stimuli, mechanical stimuli are commonly applied to the tail or paw. One of the first tests of mechanical nociception still used in laboratories was developed by Randal and Selitto (Randall and Selitto, 1957) and involves the application of increasing measurable force to the tail or hindpaw. This force is continuously increased until a

paw/tail withdrawal or vocalization is recorded and the weight of the force to obtain the nocifensive response (in grams) is recorded.

First developed over 100 years ago to test touch sensibility in man (Fruhstorfer et al., 2001), Von Frey filaments are still used in laboratories today. The Von Frey filament test involves manually applying a short calibrated monofilament to the hindpaw of a rodent. Increasingly stronger filaments are applied until a nocifensive paw withdrawal response is observed. An electronic version of Von Frey filaments is also used and involves using a single filament tip on a force transducer that measures the amount of force applied to a rodent's paw when withdrawal is observed (Cunha et al., 2004).

1.3.1.3 Chemical:

The use of chemical stimuli differs from mechanical and thermal stimuli in that the application of pain causing chemicals commonly induces a slow, long lasting nociceptive response that cannot be immediately removed. The most common chemical substance used for inducing acute chemical pain is capsaicin. Capsaicin is commonly injected into the plantar surface of the hind paw and licking, biting, and flinching behaviours are measured for 5 minutes following the application (Boyce-Rustay et al., 2010). Because capsaicin can elicit a long-lasting nociceptive response, it is commonly used in conjunction with measurement of mechanical and thermal stimuli (Le Bars et al., 2001).

While the observation of pain-like behaviours in response to noxious stimuli allow for the rapid screening of newly developed compounds for analgesia, it allows little opportunity for the study of pain at the cellular level and the development of new analgesic targets.

1.3.2 Cellular

The vast majority of research into the cellular mechanisms of nociception, including the assessment of specific ion channels involved in chemical, mechanical, thermal transduction as well as action potential generation, are based on experiments using heterologous expression systems and *in vitro* preparations (Zimmermann et al., 2009). The advantages and disadvantages of these *in vitro* preparations are highlighted below.

1.3.2.1 Cultured DRG/TRG

Since the inception of tissue culture in 1907 (Harrison, 1907) neuronal cell cultures from various animals have been widely and successfully exploited as a powerful tool to answer relevant questions of neurobiology and neurodevelopment, such as neurite/axon formation and elongation, synapse formation and synaptic properties, neurotrophic factor trafficking and signaling, neurotransmitter release, electrical signaling, and intracellular protein trafficking (Melli and Hoke, 2009).

The cell bodies of nociceptors exist within the dorsal root ganglion and trigeminal ganglion and can be cultured. The advantages to using *in vitro* primary DRG or TRG cultures in the study of nociception is that it allows one to study whether a given compound has a direct effect on a primary sensory neuron, the precise monitoring of the concentrations of a given compound the primary sensory neurons are exposed to and finally, the ability to study cellular mechanisms of excitation and neurotransmitter release that would be otherwise be difficult to study *in vivo* (Burkey et al., 2004).

Of course the use of cultured sensory neurons comes with its limitations; without the surrounding tissue it is difficult to ascertain the physiological functions of the peripheral sensory neuron from its cultured somata and culture conditions frequently offer additional variables that

can alter expression of proteins or transmitters that limit extrapolation of results to the *in vivo* conditions. To overcome altered protein or transmitter expression experimenters can use acutely dissociated TRG and DRG instead of primary cultured neurons, which involve using the dissociated DRG or TRG neurons the day they are removed from the animal as opposed to after exposure to culture conditions for days/weeks.

Although much of the understanding of nociceptive transduction has come from the use of cultured or dissociated DRG/TRG somata, there is a high probability that significant quantitative differences exist in the patterns of expression of ion channels and signaling systems between the somata and the peripheral processes of the nociceptor neuron, which have often been overlooked (Kostyuk and Kostyuk, 2009), highlighting the need for an *in vitro* preparation that looks specifically at the tiny peripheral terminals.

1.3.2.2 Reprogrammed cells

A significant limitation of nociceptive research, particularly at the cellular level, is that it is almost exclusively performed in other types of mammals, the majority in rodents. Although it is possible that nociceptive transduction processes are homologous across different orders of mammals, there exists a likelihood that ion channel expression and signaling mechanisms vary.

The recently developed ability to differentiate human pluripotent stem cells and perform lineage reprogramming of human cells allows researchers to derive a wide range of neuronal subtypes, which has recently been applied to the development of human-derived nociceptors.

In 2012, researchers were able to take human pluripotent stem cells and through small molecule pathway inhibition, convert these cells to neurons expressing canonical markers and functional properties of human nociceptors (Chambers et al., 2012) and earlier in 2015 two

separate research groups were able to reprogram human fibroblasts into noxious stimulus-detecting (nociceptor) neurons (Blanchard et al., 2015; Wainger et al., 2015).

Although these techniques are thought to overcome the interspecies differences that may exist when studying nociception and reduce the use of animals necessary for culturing DRG or TRG's, they still focus on the cell somata and the use of a variety of pathway activators/inhibitors as well as various culture conditions during the creation of these reprogrammed nociceptors may possibly leave these cells with less resemblance to human cells than researchers would hope for.

1.3.2.3 *In vitro* corneal preparations

The cornea is one of the most densely innervated tissues in the body and is richly supplied by sensory and autonomic nerve fibres (Muller et al., 2003). The majority of sensory neurons in the cornea are axons whose cell bodies reside in the ophthalmic division of the trigeminal nerve and predominately sense pain (Al-Aqaba et al., 2010). Because of the high density of nociceptive terminals within the cornea, *in vitro* electrophysiology and Ca^{2+} imaging preparations for studying signaling in the terminals have been developed.

The nociceptor peripheral terminals in the cornea terminate in the most superficial layer of the corneal epithelium in most species. This arrangement has allowed researchers to create an *in vitro* preparation in which the ciliary nerve of the eye is electrically stimulated and a recording electrode is applied to the corneal surface with light suction to record antidromic signals arriving in the endings of the nerve terminals (Al-Aqaba et al., 2010). While this preparation has provided insight into impulse generation in nerve terminals, especially in response to cold stimulation (Carr and Brock, 2002; Carr et al., 2002; Carr et al., 2003; Madrid et al., 2006), it does not allow

for activation of the peripheral terminals themselves, leaving this preparation restricted to the study of efferent signaling in nociceptors.

A second *in vitro* corneal preparation involves using a large electrical stimulation across the entire eye, paired with Ca^{2+} imaging in the peripheral terminals (Gover et al., 2003; Gover et al., 2007). This preparation appears to be restricted to the observation of Ca^{2+} handling in the peripheral terminals only, since the large stimulation used does not appear to evoke single, rapid and repeatable Ca^{2+} transients as would be seen if single action potentials were elicited by the electrical stimulation.

Aside from the inability to directly activate single nociceptors at the peripheral terminals, the *in vitro* corneal preparations cannot be used to study nociceptive states such as neurogenic inflammation and peripheral sensitization due to the lack of immune cells and vasculature in the cornea (Niederkorn, 2005).

1.3.2.4 Isolated skin-saphenous nerve preparation

An *in vitro* model that allows for the study of responses of primary sensory neurons in the rodent skin is the saphenous skin–nerve preparation. This preparation involves dissecting the saphenous nerve and a large part of the hindpaw skin innervated by the saphenous nerve from a rodent and superfusing it with a physiological solution (Reeh, 1986). This preparation enables extracellular recording of propagated action potentials downstream from the receptive fields of single sensory nerve endings in the skin in response to electrical, thermal, mechanical and chemical stimuli (Zimmermann et al., 2009).

While this preparation is one of few that activates peripheral terminals of nociceptors and observes axons as opposed to cell bodies, it does have its limitations. A major limitation of the preparation is that a lot of patience and time is required to find and record from single units;

teasing apart the saphenous nerve to not only record from a single primary afferent but to confirm that the afferent being recorded from is a nociceptor is tremendously difficult (Zimmermann et al., 2009).

Although this preparation does activate the peripheral terminals, recordings are taken a large distance away from the stimulus allowing only for the study of the action potentials as a result of the stimulus at the terminals, the inability to directly study the physiological processes that are occurring at the terminals themselves and the impossibility of studying signaling within the terminals.

The *in vitro* preparations that currently exist for studying nociception have been key in the current understanding of nociception and development of analgesic targets, but the development of novel methods, particularly those that more closely reflect *in vivo* conditions and allow the direct study of the elusive tiny pain-sensing terminals are necessary in moving pain research forward.

1.4 Calcitonin gene-related peptide

The bulk of this thesis focuses on CGRP-containing nociceptors due to their involvement in numerous nociceptive processes highlighted below. Calcitonin gene-related peptide (CGRP) is a vasodilator neuropeptide found in a subpopulation of peptidergic nociceptive neurons (Benarroch, 2011). Within these small nociceptors it exists in two isoforms (α and β) that although being of similar homology are produced by different genes with the α-isoform being found in much higher levels (Seybold, 2009). CGRP is located within the peripheral terminals of nociceptors, packaged in large dense core vesicles, where its release contributes to neurogenic inflammation (Benemei et al., 2009; Kilo et al., 1997; Richardson and Vasko, 2002). CGRP released at the central terminals within the dorsal horn of the spinal cord or trigeminal nucleus caudalis has two main functions, one acting back on nearby central terminals to facilitate neurotransmitter release (Kangrga et al., 1990; Kangrga and Randic, 1990) and secondly acting on spinal neurons to increase glutamate activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and N-methyl-D-aspartate (NMDA) receptors (Ebersberger et al., 2000; Seybold, 2009).

1.4.1 CGRP receptor

Functioning CGRP receptors are composed of a G-protein coupled receptor, calcitonin-like receptor (CLR) paired with a single transmembrane domain protein called receptor activity modifying protein type 1 (RAMP1) that traffics the CLR to the cell membrane surface and a receptor component protein (RCP) that determines which G-protein is coupled to the receptor (Figure 1.3) (Durham and Vause, 2010).

CGRP receptors are found in numerous pain-related locations and cell types throughout the body where their activation leads to pro-nociceptive effects. The CGRP receptor is localized

to vascular smooth muscle (Brain and Grant, 2004), central terminals of non-CGRP containing nociceptors (Eftekhari and Edvinsson, 2010; Natura et al., 2005), mast cells (Lennerz et al., 2008), satellite glia and Schwann cells (Eftekhari et al., 2010), as well as in second order nociceptive neurons within the spinal cord and trigeminal nucleus caudalis (Eftekhari and Edvinsson, 2010). Release of CGRP and activation of its receptor are involved in a variety of nociceptive states including central sensitization, neurogenic inflammation and most importantly migraine (Benarroch 2011).

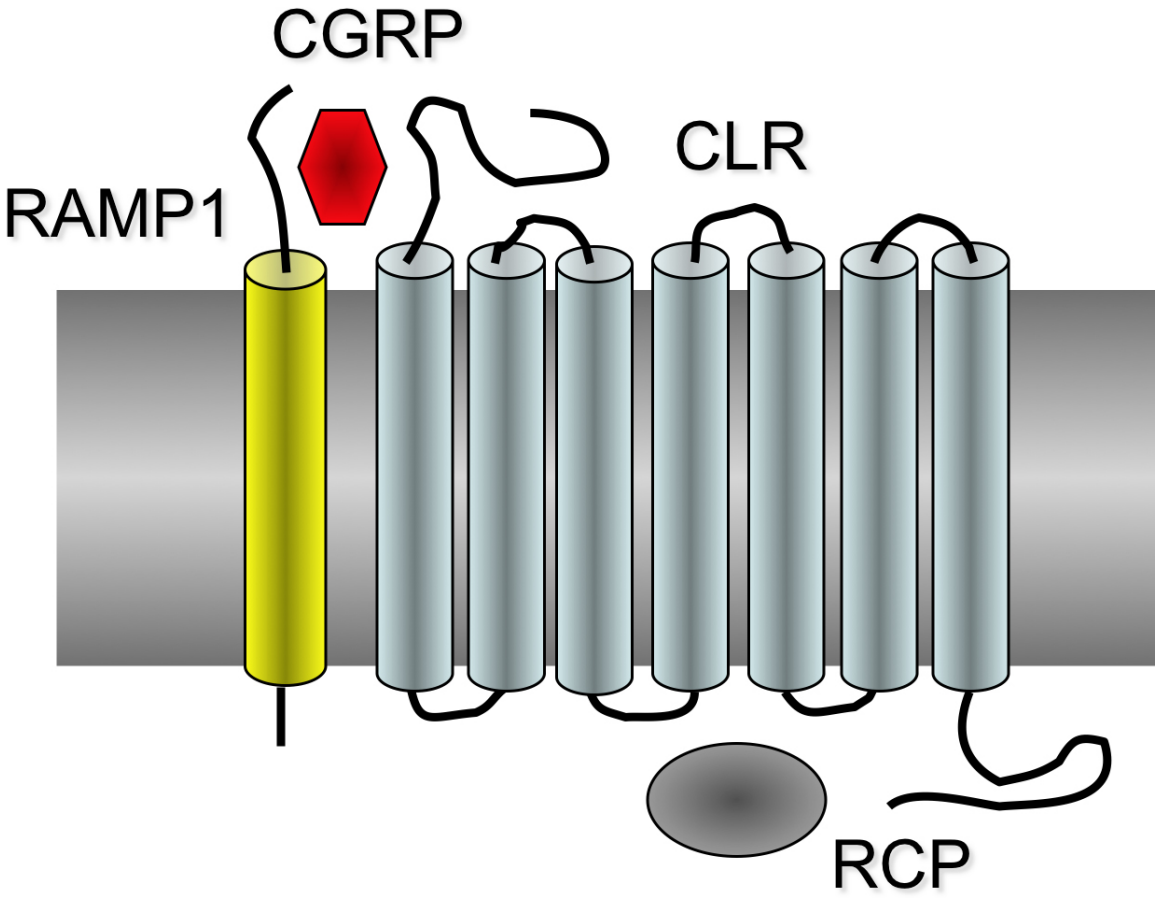

Figure 1.3. The functional CGRP receptor.
The CGRP receptor consists of three subunits; the calcitonin-like receptor (CLR), the transmembrane domain protein called receptor activity modifying protein type 1 (RAMP1) and a receptor component protein (RCP). Adapted from (Recober et al., 2009)

1.4.2. CGRP and central sensitization

Central sensitization is described as an enhancement in the function of neurons and circuits in nociceptive pathways caused by increases in membrane excitability and synaptic efficacy within the spinal cord (Latremoliere and Woolf, 2009). Central sensitization can be initiated by damage to peripheral nerves or by repeated exposure to particularly intense noxious stimuli and coincides with spontaneous pain, pain elicited by normally innocuous stimuli (allodynia) or an exaggerated or prolonged response to a noxious stimulus (hyperalgesia) (Ji et al., 2003).

While glutamate has been indicated as the small molecule neurotransmitter responsible for rapid excitatory synaptic transmission between nociceptors and dorsal horn neurons, it has been noted that activation of C nociceptors, which contain a wide variety of neuropeptides, is necessary to induce central sensitization (Cook et al., 1987).

While CGRP on its own is incapable of directly increasing synaptic release from nociceptors or directly increasing excitation of dorsal horn neurons to cause central sensitization (Gamse and Saria, 1986), it is thought to play an indirect role in the induction and maintenance of central sensitization (Seybold, 2009).

During induction of central sensitization activation of CGRP receptors in dorsal horn neurons has been shown to increase the excitability of spinal neurons (Bird et al., 2006), likely through phosphorylation of AMPA receptor subunits, increasing their insertion into the postsynaptic plasma membrane (Esteban et al., 2003). CGRP is also thought to contribute to the induction of central sensitization by competing with substance P for catabolism by endopeptidases (Le Greves et al., 1985). Substance P is another neuropeptide that is commonly co-expressed and released with CGRP in small diameter nociceptors (Lundberg et al., 1985).

Substance P binds to neurokinin 1 (NK1) receptors, another G-protein coupled receptor, on the secondary spinal neurons resulting in the phosphorylation of NMDA and AMPA receptors leading to increased ion conductance also necessary for the induction of central sensitization (Latremoliere and Woolf, 2009).

Intrathecal administration of a CGRP receptor antagonist blocks mechanically-evoked nociceptive responses in animals after the development of hyperalgesia indicating that activation of CGRP receptors is also necessary in the maintenance of central sensitization (Sun et al., 2004).

1.4.3. CGRP and neurogenic inflammation

The process of inflammation is classically defined as involving four cardinal signs: dolor (pain), calor (heat), rubor (redness) and tumor (swelling) (Scott et al., 2004) with pain previously being thought simply as a symptom of inflammation and not actively taking part in the process. More recently it has been discovered that nociceptors, specifically peptidergic nociceptors, actively take part in the process of inflammation. Nociceptors are thought to not only provide orthodromic signaling to the CNS, but action potentials also signal antidromically at branch points back down to the periphery, a process referred to as the axon reflex (Figure 1.4) (Chiu et al., 2012). The action potentials traveling back to the periphery are believed to lead to a local and rapid release of neural mediators from both peripheral axons and terminals contributing to the calor, rubor and tumor of inflammation, a process now referred to as neurogenic inflammation.

Neurogenic inflammation is primarily initiated by the release of CGRP and substance P (Richardson and Vasko, 2002), which act directly on the endothelial and smooth muscle cells of the vasculature with CGRP causing vasodilation (McCormack et al., 1989) and substance P

increasing the permeability of capillaries leading to plasma extravasation (leakage) and edema (Saria, 1984).

The release of CGRP and substance P along with other peptides further contribute to neurogenic inflammation by attracting and activating neutrophils, macrophages and lymphocytes at the site of injury, causing the degranulation of mast cells as well as dendritic and T-cell priming (Ansel et al., 1993; Ding et al., 2008; Rochlitzer et al., 2011). Recent findings support the idea that peptidergic nociceptors not only play a passive role in host defense by detecting noxious stimuli and initiating avoidance behaviour, but also play an active role via the immune system to modulate the responses to and combat of harmful stimuli (Chiu et al., 2012).

During this inflammatory process the variety of mediators released from other cell types cause a peripheral sensitization of the nociceptors lowering the excitation threshold leading to peripherally-mediated hyperalgesia and/or allodynia (Schaible, 2007).

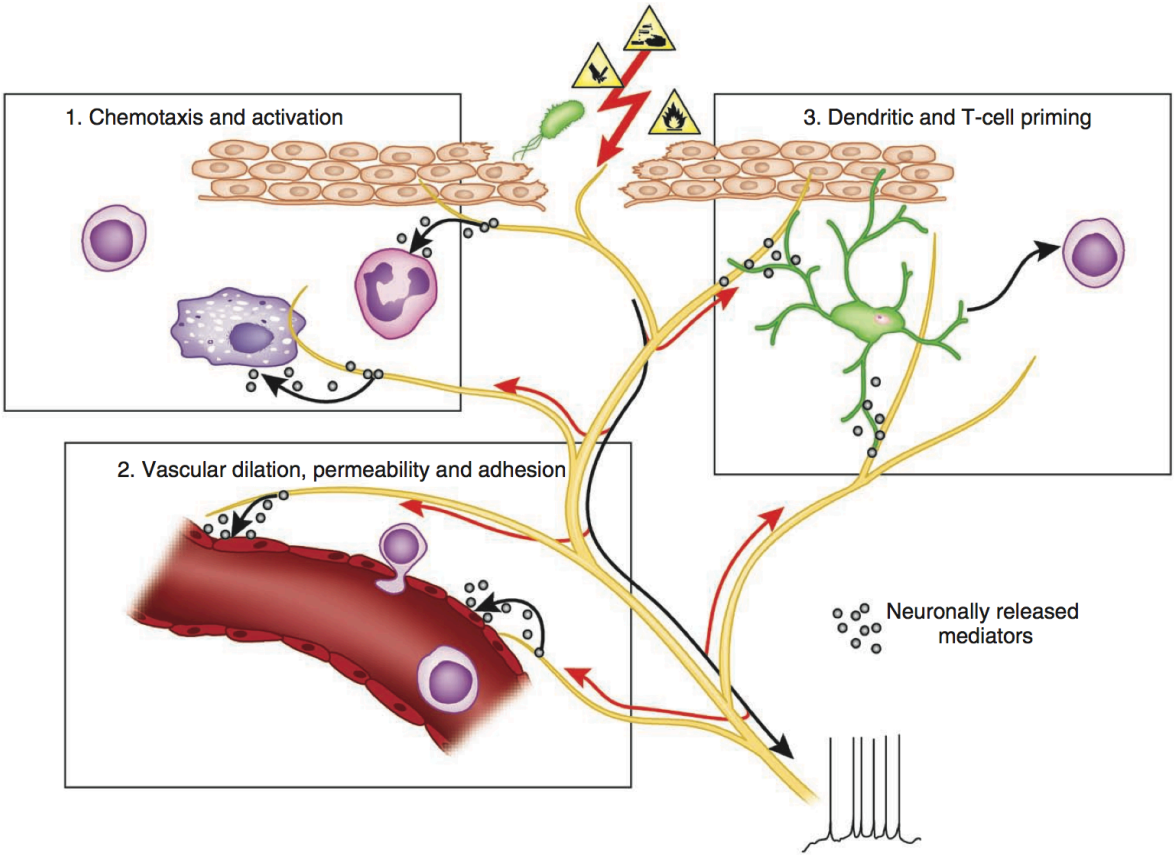

Figure 1.4. CGRP and neurogenic inflammation.
The release of CGRP and other neuropeptides from efferent signaling in nociceptors leads to neurogenic inflammation through: **1**) attracting and activating neutrophils, macrophages and lymphocytes at the site of injury, and causing the degranulation of mast cells. **2**) Vasodilation, plasma extravasation and edema. **3**) dendritic and T-cell priming. Adapted from (Chiu et al., 2012)

1.4.4. CGRP and Migraine

Migraine is considered a neurovascular disorder characterized by a severe, debilitating and throbbing unilateral headache associated with anorexia, nausea, vomiting, photophobia, phonophobia and/or diarrhea (Goadsby et al., 2002). While the initial trigger for a migraine attack remains unknown, it is largely accepted that activation of the nociceptors innervating the meninges is necessary (Ebersberger, 2001; Pietrobon and Striessnig, 2003). It has been previously hypothesized that migraine pain was due to vasodilation of blood vessels of the cerebrum and that CGRP released from trigeminal peptidergic nociceptors causes this dilation (Jansen-Olesen et al., 1996).

The vasodilation that occurs during migraine is no longer believed to be the cause but rather a secondary affect that may contribute to the painful state of migraine (Burgos-Vega et al., 2015). Despite this, CGRP is still thought to play an important role in the pathophysiology of migraine for several reasons: CGRP levels are elevated during a migraine attack (Goadsby et al., 1990), migraine patients that are infused with CGRP develop a delayed headache, fulfilling the criteria of a migraine (Lassen et al., 2002), the administration of medications during a migraine attack reduce CGRP levels that correspond with relief (Stepien et al., 2003) and finally the success of CGRP receptor antagonists in ameliorating a migraine attack all point towards the involvement of CGRP in migraine (Villalon and Olesen, 2009).

The two possible sites of action CGRP may take in the contribution to migraine are at the level of the meninges where it is released by the peripheral terminals of nociceptors or at the central terminals within the spinal trigeminal nucleus (Burgos-Vega et al., 2015). In the meninges it is possible that CGRP release results in a state of neurogenic inflammation,

peripheral sensitization and allodynia causing meningeal nociceptors to react to normally non-painful vascular stimuli (Olesen et al., 2009).

Centrally, it is thought that the high frequency of action potential firing from the peripheral nociceptors to the trigeminal nucleus caudalis as a result of peripheral sensitization can lead to increased CGRP release centrally and the development of central sensitization as described above, contributing to the painful phase of the migraine attack (Dodick and Silberstein, 2006).

The development of a preparation to study peripheral CGRP fibres may help identify the possible underlying mechanisms of activation and peripheral sensitization and may have important implications for understanding and mitigating the pathogenesis of migraine headaches.

1.5 Triptans and Migraine

While the etiology of migraine headache is poorly understood, sumatriptan and the triptan class of serotonin receptor subtype-selective drugs have an established efficacy in treating the pain associated with migraine. Triptans are a class of tryptamine-based drugs that activate serotonin type 1B and D ($5\text{-}HT_{1B/D}$) G-protein coupled receptors. It has been long debated whether the anti-migraine actions of triptans occurs via activation of 5-HT1 receptors on arterial smooth muscle, the peripheral terminals of nociceptors, or within the nociceptive system of the CNS (Hoskin and Goadsby, 1998; Humphrey and Goadsby, 1994)

1.5.1 Vasculature

Classically the effectiveness of triptans in the treatment of migraine pain has been attributed to their vasoconstrictive properties, which have been shown to occur exclusively through activation of $5\text{-}HT_{1B}$ receptors on cranial arterial smooth muscle (Razzaque et al., 1999). But because vascular changes seen with triptan usage have not temporally coincided with pain relief (Limmroth et al., 1996) it is not known if vasculature 5-HT1B receptor activation is necessary for migraine relief.

1.5.2 Central nociceptive system

Activation of dural afferents can result in central sensitization within the trigeminal nucleus caudalis (TNC) that may play a role in the pathogenesis of migraine (Dodick and Silberstein, 2006). While sumatriptan had been proposed to result in migraine relief through central mechanisms, this seems unlikely due to its hydrophilic nature, resulting in poor penetration of the blood brain barrier (Kaube et al., 1993; Sleight et al., 1990). More lipophilic triptans have been shown to have central effects, blocking dura-evoked activation of TNC neurons in both electrophysiological and c-*fos* studies (Cumberbatch et al., 1997; Hoskin and

Goadsby, 1998) indicating that triptans may possibly act centrally, particularly if there is a disruption in the blood brain barrier during migraine, which has been proposed to occur (Moskowitz and Cutrer, 1993).

1.5.3 Peripheral nociceptors

It is widely recognized that activation of dural nociceptive afferents is responsible for the development of migraine headache (Ebersberger, 2001; Pietrobon and Striessnig, 2003) and these dural afferents seem a likely target for triptans in the relief of migraine. 5-HT_{1D} receptors have been found to be localized on peptidergic nociceptors (Potrebic et al., 2003) and it is proposed that triptans mediate their anti-migraine effects through inhibition of peptidergic dural afferents (Ahn and Basbaum, 2005). Activation of nociceptors in the dura mater triggers neurogenic inflammation as described above and it has been proposed (in the somata of nociceptors), that sumatriptan inhibits CGRP and presumably substance P release through the inhibition of a variety of Ca^{2+} channels (P/Q, L and N-type) (Xiao et al., 2008). The inhibition of Ca^{2+} influx and subsequent decrease in neuropeptide release is thought to likely result in the resolution of neurogenic inflammation. While mechanisms of sumatriptan's actions have been proposed in the cell bodies of nociceptors as well as the central terminals (Arvieu et al., 1996), a study has yet to look at the effect sumatriptan has in the peripheral terminals of nociceptors.

1.6 Peripheral μ-opioid receptors

Opioids have been used for hundreds of years for the treatment of pain and are still considered to be the gold standard for the treatment of severe pain and inflammation (Stein and Kuchler, 2012). The analgesic effects of opioids occur via activation of opioid receptors (μ, δ, or κ) which are G-protein coupled receptors (mainly G_i/G_o) that have been found to reduce neuronal activity by inhibiting adenylyl cyclase, decreasing the conductance of voltage gated Ca^{2+} channels and/or opening rectifying K^+ channels in many neuronal systems (Stein and Zollner, 2009).

Although opioids are very effective analgesics, their use is hampered by side effects such as depression of breathing, nausea, clouding of consciousness, constipation, addiction and tolerance (Kieffer and Gaveriaux-Ruff, 2002).

Traditionally the analgesia experienced with the use of opioids was believed to occur mainly via inhibition of the CNS and although peripheral anti-nociceptive effects were believed to exist with opioid use more than a century ago (Stein, 1993), it wasn't until the 1980's that evidence began to surface that opioid receptors did indeed exist outside the CNS in peripheral sensory neurons (Stein, 1995; Stein et al., 1990).

All three of the main types of opioid receptors have been found to exist within small-, medium-, and large-diameter DRG neurons (Buzas and Cox, 1997; Chen et al., 1997) and have been shown to be intra-axonally transported into the neuronal processes (Fields et al., 1980; Young et al., 1980) of the peripheral nerve terminals of rodents (Stein et al., 1990) and humans (Stein et al., 1996).

Of the three types of opioid receptors, agonists of the μ-opioid receptor remain the most effective analgesics and in the cell bodies of sensory neurons their main inhibitory effect is

believed to occur via inhibition of various Ca^{2+} channels (Stein and Zollner, 2009) and not through the modulation of K^{+} channels (Akins and McCleskey, 1993). Although μ-opioid receptor activation in the cell bodies has also been shown to inhibit TRPV1 channels (Endres-Becker et al., 2007) as well as activate one subtype of G-protein coupled inward-rectifying potassium channel (GIRK2) (Nockemann et al., 2013).

While inhibitory mechanisms of μ-opioid receptor activation identified in the cell bodies of nociceptors provides valuable insight, the possibility exists that ion channel prevalence and density differ in the peripheral terminals and therefore the mechanisms by which μ-opioid agonists cause analgesic and/or anti-inflammatory effects in the pain-sensing terminals remain to be established. Identification of mechanisms by which μ-opioid receptor activation leads to analgesia in the peripheral terminals of nociceptors will aid in the targeting and development of peripherally restricted opioids resulting in analgesia with greatly improved side effect profiles (Sehgal et al., 2011).

1.7 General hypothesis

The general hypothesis of this thesis is that the intracranial meninges of mice can be used to create a novel ex vivo preparation that allows the study of nociceptive signaling within the peripheral terminals of selectively identified dural nociceptors and that this preparation will generate novel insights into the mechanisms of peripheral analgesia.

1.8 Thesis objectives

1) Create an *ex vivo* preparation that allows for the study of nociceptive signaling within the peripheral terminals of nociceptors (Chapter 2).

2) Provide direct evidence of the axon reflex in an individual branching nociceptor (Chapter 2).

3) Study the effect 5-$HT_{1B/D}$ receptor activation has on action potential-evoked Ca^{2+} transients in peripheral CGRP nociceptive terminals (Chapter 3).

4) Identify the anti-nociceptive mechanism(s) of μ-opioid receptor activation in peripheral CGRP nociceptive terminals (Chapter 4).

5) Extend *ex vivo* findings from aim (4) *in vivo* using a behavioural model of nociception and a novel peripherally restricted μ-opioid agonist (Chapter 4).

CHAPTER 2

Functional imaging within individual pain fibres *ex vivo* with optical microscopy

2.1 ABSTRACT

Here I introduce a simple experimental approach for studying afferent pain fibre physiology. I have developed a mouse *en bloc* dural–skull preparation for optical microfluorometric imaging to directly study the physiological functioning in selectively identified, individual nociceptive fibre free nerve endings. Functional optical imaging using widefield epifluorescence microscopy was combined with electrophysiological stimulations, pharmacological manipulations, and the UV photolysis of caged compounds. For the first time, I show high–resolution functional imaging of single action potential–evoked fluorescent transients, as well as sub– and supra–threshold calcium signaling events within individual nociceptive fibre terminations. This novel experimental approach opens up a new window for studying nociceptive fibre physiology and pathophysiology.

2.2 INTRODUCTION

Our current understanding of peripheral pain fibre physiology has been almost exclusively derived from research in which electrophysiological recordings were made in the somata of cultured or acutely dissociated cell preparations taken from sensory ganglion cells – not from the afferent fibre terminations themselves (Kostyuk and Kostyuk, 2009; Woolf and Ma, 2007). Investigating afferent signaling selectively within pain fibres is challenging because the majority of nociceptors are thin unmyelinated axon fibres that are inaccessible with conventional electrophysiological techniques such as intracellular recording with patch-clamp pipettes. As well, the unmyelinated nociceptive fibres are characteristically located in highly light scattering tissues that are not compatible with functional optical imaging.

Pain sensitivity within the skull is restricted to the intracranial meninges, the system of membranes that envelops the brain. Afferent thinly myelinated Aδ-fibres and unmyelinated C-fibres of the trigeminal nerve that originate primarily from neurons in the ophthalmic division of the trigeminal ganglion densely innervate the meninges, in particular the cranial dura mater (Fricke et al., 2001). Trigeminal innervation of the dura mater serves an exclusive nociceptive function (Strassman and Levy, 2006) and despite species differences in brain size and organizational complexity, appears remarkably similar across mammals from mice and rat to cat and human (Feindel et al., 1960; Leiser and Moxon, 2007; Recober et al., 2009). I have developed a mouse *en bloc* dural–skull preparation that maintains individual nociceptive fibre integrity intact for *millimeters* and offers unprecedented tissue properties for optical imaging in an intact preparation. Here I introduce a simple imaging approach for studying afferent pain fibre physiology using widefield epifluorescence microscopy employing standard optical equipment that is widely available to most laboratories.

2.3 MATERIALS AND METHODS

2.3.1 Dissection.

This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Mice (25 wild type; 20 Transgenic *Tg* [*Calca*–EGFP] (*GENSAT Project at Rockefeller University*) (1 month – adult) were anaesthetized by intraperitoneal injection of urethane and intracardially perfused with ice – cold physiological solution consisting of (in mM): 125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 10 Glucose, 2 $MgCl_2$, 1.25 NaH_2PO_4, and 2 $CaCl_2$. The head was removed from the body at the atlanto–occipital joint. The skin was cut along the skull sagittally and gently moved laterally on both sides. Dissection scissors were then inserted into the foramen magnum and the skull with brain was cut laterally below the occipital and parietal bones on both sides. The two lateral cuts were joined sagitally at the ventral section of the frontal bone. The remaining skull and brain was placed in a bath of physiological solution and the brain carefully removed from the skull leaving intact dura mater and arachnoid mater layers attached to the skull. The interparietal bone was removed by cutting along the lambdoidal suture and the frontal bone removed by cutting along the coronal suture. Two separate complete parietal bone – dural layer preparations were obtained with a remaining cut along the sagittal suture. Individual dural–skull preparations were placed dural layer up in a microscope chamber and perfused with physiological saline, gravity fed at a flow rate of 3 ml/min and maintained at either 25 ± 0.5 or 35 ± 0.5 ^{o}C with an inline heater (*Warner Instruments*).

2.3.2 Calcium indicator application.

Afferent nociceptive fibres were selectively loaded with the membrane permeant high affinity Ca^{2+} indicator Rhod–2 AM (Invitrogen) by applying a small piece of indicator solution

soaked Surgifoam® to the rostral area of the preparation for 10 – 20 seconds. The 1mM indicator solution was made up fresh each day in physiological solution containing 5% Pluronic F–127/DMSO.

2.3.3 Widefield epifluorescence microscopy.

I used the X–Cite 120PC system (*EXFO Electro–Optical Engineering Inc.*) light source (12% intensity) directly coupled to an Olympus BX51WIF upright research microscope by a 3 m liquid light guide. The light intensity was attenuated with two neutral density filters in series (Olympus N.D.25 and N.D.6) and shutter controlled with a ProScan II Controller (*Prior Scientific*). The illumination and fluorescence light was filtered using either a TRITC filter set (41002 Olympus BX2 mounted -HQ535/50x HQ610/75m Q565LP), a FITC filter set (41001 Olympus BX2 mounted -HQ480/40x HQ535/50m Q505LP), or a Cy5™ filter set (41024 Olympus BX2 mounted -HQ620/60x HQ665lp Q660lp). Epifluorescence was detected with two high sensitivity cooled CCD cameras - the 16-bit, 512x512 ImagEM EM CCD Camera (C9100-13, *Hamamatsu*) cooled to -65°C and the 16-bit, 1344 x 1024 ORCA-R2 CCD camera (C10600-10B, *Hamamatsu*) cooled to -35°C. Images were acquired using variable frame rates, binning, and defined regions of interst.

2.3.4 UV photolysis.

Photorelease of caged compounds was achieved using a 355 nm wavelength, diode pumped solid–state laser (DPSL-355-30, *Rapp Optoelectronic GMBH*) capable of 30 mW output power and 10 kHz (3 μJ/pulse) repetition rate. UV light was coupled to the microscope using a homemade optical alignment system. The UV beam from a 100 μm core quartz light guide was directed through focusing optics and a 5x beam expander BXUV-4.0-5X-355 (*CVI Laser*) to a custom built beamsplitter (zt355/650rpc 25.5x36x1.5 mm sputtered laser polychroic, *Chroma*

Technology Corp.) that was seated at the back aperture of the objective. With this set up I achieved focal uncaging by diffraction limited spot illumination.

2.3.5 Morphological two–photon laser imaging.

I performed two–photon imaging using a custom modified Olympus BX51WIF upright research microscope interfaced with an Ultima–X-Y laser–scanning module (*Prairie Technologies Inc.)* directly coupled to a Mai Tai XF (*Spectra Physics*) mode–locked Ti: sapphire laser source. EGFP and Alexa Fluor 594 fluorophores were excited at 920 nm and 780 nm respectively. Images were acquired using high numerical aperture, long working distance, water immersion Olympus objective lenses (either a LUMPLFL 40XW/IR-2 40X or a UMPLFLN 10X) and the epifluorescence detected with two top–mounted low dark current (<10 *n*A) high sensitivity (>8500 *A*/lumen) external PMT detectors (*Hamamatsu*). Emission of the relevant wavelengths was simultaneously acquired with the use of selective emission bandpass 510 nm (40 nm bandpass) and 605 nm (55 nm bandpass) filters.

2.3.6 Electrical stimulation.

A Master 8-CP, software controlled 8-channel pulse stimulator and ISO–Flex stimulus isolator unit (*A.M.P.I.*) were used to deliver electrical stimulations via 1μm bipolar tungsten electrodes (*WPI*). Stimulation intensity was kept just above threshold to elicit action potentials (140–180 μA for a duration of 100 μs) and was not adjusted throughout the course of an experiment.

2.3.7 Immunohistochemistry.

En bloc dural–skull preparations were dissected as described above and placed in 2% paraformaldehyde for 15 minutes. Preparations were then rinsed with 1X phosphate buffered saline (PBS) 4 times for 5 minutes each before being placed in a blocking solution consisting of

10% goat serum, 1% bovine serum albumin (BSA), and 1xPBS containing 0.3% triton for 60 minutes. The primary antibody, rabbit anti–α–CGRP (*Penninsula Laboratories*), was then diluted 1:10000 with 10% goat serum, 1% BSA, and 1xPBS containing 0.3% triton and the preparations incubated at 4^{o}C overnight. The following day the preparations were then rinsed with 1xPBS 4 times for 5 minutes each. The secondary antibody, Alexa Fluor 594 goat anti–rabbit IgG (*Invitrogen*), was diluted to 1:100 with 1×PBS containing 0.3% triton and the preparations incubated at 4^{o}C for 1 hour. The preparations were then rinsed 4 times for 5 minutes each and then viewed with epifluorescence and two–photon microscopy.

2.3.8 Drugs.

Tetrodotoxin (1μM) (*Alomone Labs*), capsazepine (10μM) (*Sigma*) and the selective TRPV1 custom caged molecule BCMACMOC–caged capsaicin (1μM) were bath applied. For Ca^{2+} photolysis experiments, dural–skull preparations were incubated for 10 minutes in physiological solution containing the cell permeant form of the Ca^{2+} cage *o*–nitrophenyl EGTA, AM (NP–EGTA, AM) (*Invitrogen*) at 30μM and 0.5% DMSO. The myelin stain FluoroMyelin Red® (*Invitrogen*) was bath applied (10 ml), diluted from the stock solution 100×. Isolectin GS-IB4 from *Griffonia simplicifolia*, AlexaFluor™ 647 conjugate (*Invitrogen*) was diluted to 1.5 μg/25 ml and bath applied.

2.4. RESULTS

2.4.1 *En bloc* dural–skull preparation

The cranial dura mater consists of two layers: an outer periosteal layer that lines the internal surface of the cranial cavity and an inner meningeal layer adjacent to the arachnoid membrane that borders the cerebrospinal fluid-filled subarachnoid space. I find that careful removal of the brain leaves behind intact dura and arachnoid mater layers attached to the skull along with nociceptive fibres embedded in the periosteal layer of the dura that run adjacent to the calvaria (Figure 2.1). Periosteal nociceptive fibres were selected for experimentation by removing complete parietal bones with intact dural layers from the mice and placing the *en bloc* dural–skull preparations dural layer up in an upright research microscope perfusion chamber (Figure 2.2A–D). Individual nociceptive fibre integrity is preserved intact for *millimeters*, well protected on one side by the calvaria and by the dural layers on the other. The thinness of the mouse dural layer (~10 μm) that is maintained over the expanse of the parietal skull bone (~5 × 5 millimeters) offers unprecedented tissue properties for optical imaging in an intact preparation and offers a unique opportunity for studying the physiology of afferent pain fibres (Figure 2.3A and B).

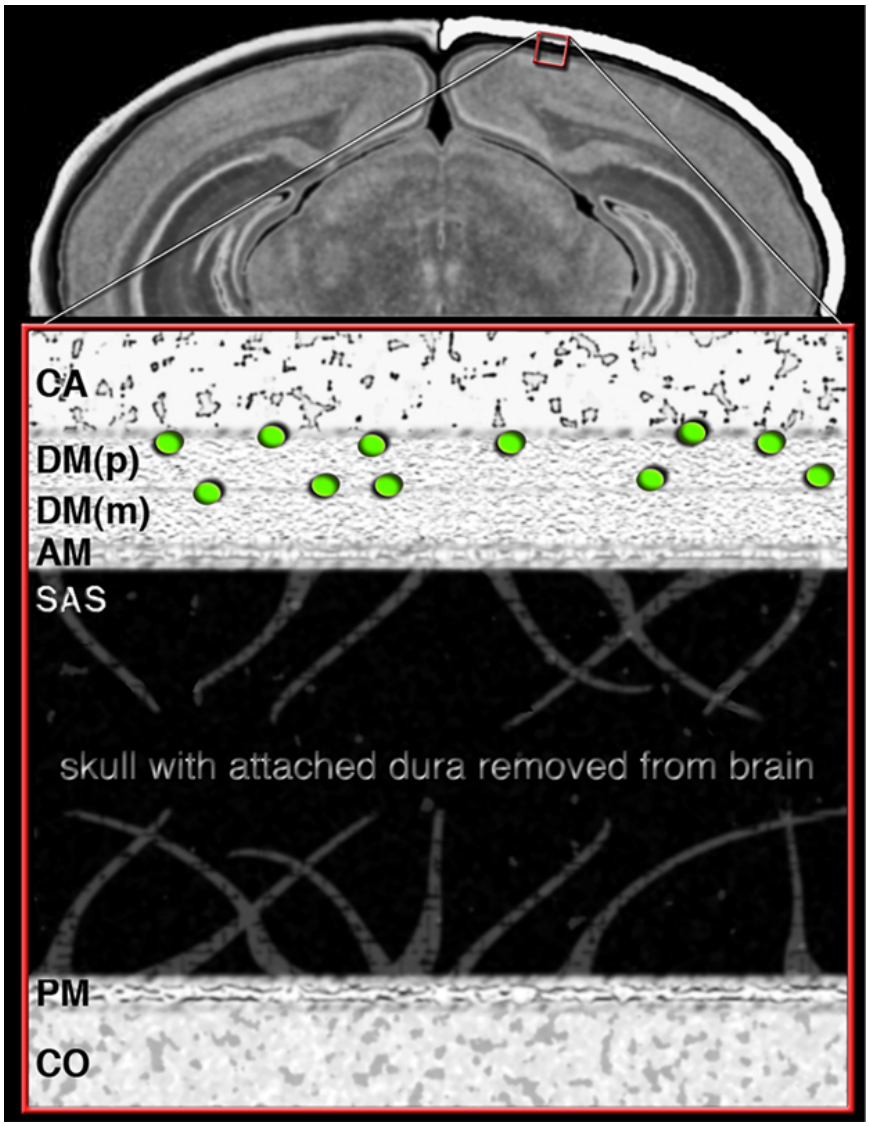

Figure 2.1 Nociceptive innervation in the cerebral dura mater.
Boxed area in the coronal image of a mouse brain and skull marks the zoomed up area below showing the structural organization of the meninges and highlights the nociceptive fibres (green circles) protected by the calvaria above and the dural layers below. The brain is removed at the level of the subarachnoid space, with broken arachnoid trabeculae shown. CA; calvaria, DM(p); periosteal dura mater, DM(m); meningeal dura mater, AM; arachnoid mater, SAS; subarachnoid space, PM; pia mater, CO; cortex.

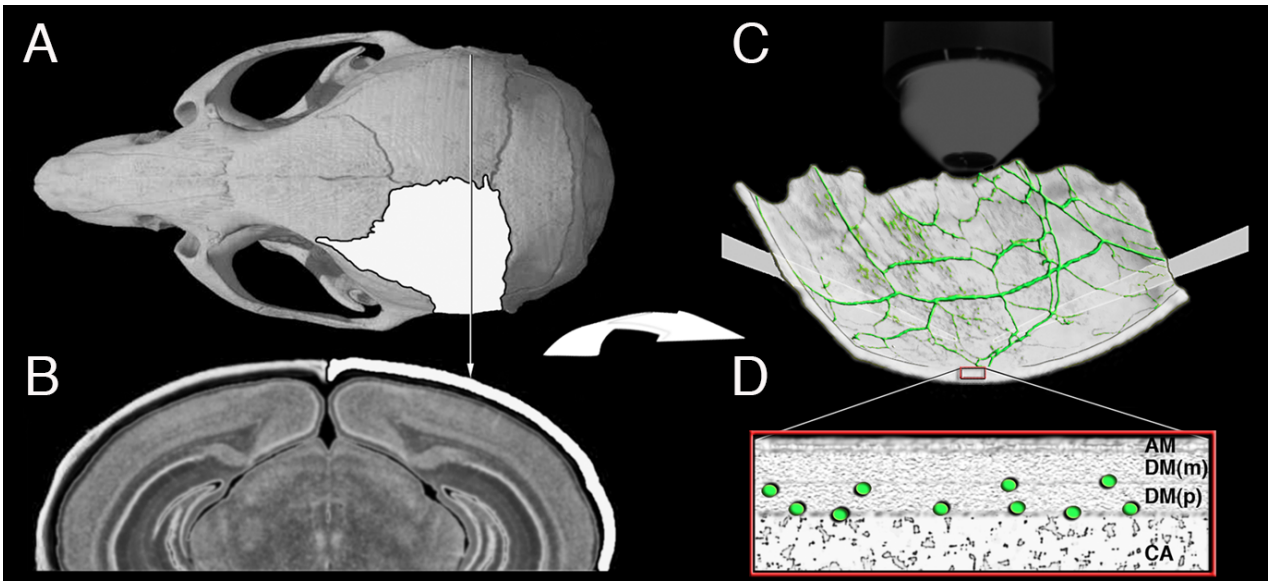

Figure 2.2. *En bloc* dural–skull preparation for imaging nociceptive fibre free endings. **A**) The complete parietal skull bone used in the dural–skull preparation is highlighted in white in this mouse skull image. The arrow marks the anatomical location of the coronal section (**B**). **C**) The ventral aspect of the dural layers allows for optical imaging of the nociceptive fibres as well as simultaneous micropipette access during experiments that may involve electrical stimulation as well the pressure injection of drugs. **D**) The boxed area taken from (**C**) shows the structural organization of the dural layers and highlights the nociceptive fibres for imaging (green circles); CA; calvaria, DM(p); periosteal dura mater, DM(m); meningeal dura mater, AM; arachnoid mater.

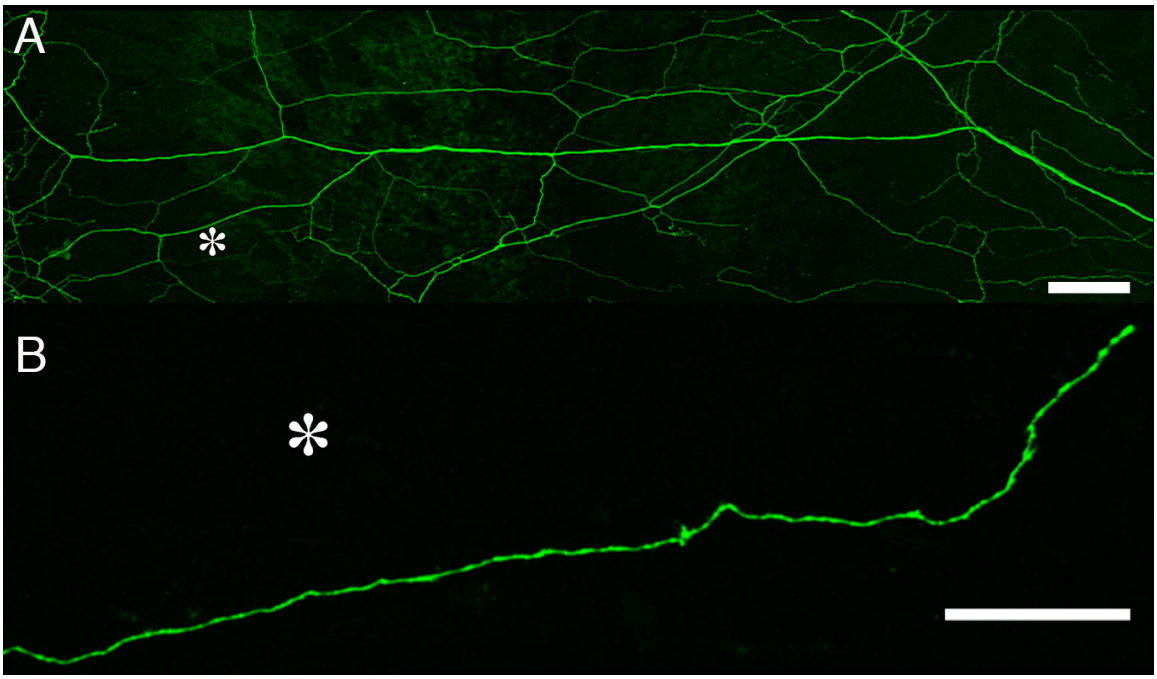

Figure 2.3. CGRP innervation of the dura.
A) Sequential images taken across a portion of the preparation highlight the dense network of CGRP nociceptive fibres. The asterisk highlights the location of the terminal fibre shown in (**B**) at higher magnification. Scale bars; **A**) 300 μm; **B**) 50 μm

2.4.2 Selective nociceptive fibre identification

It is common practice to classify nociceptive neurons depending on whether they contain peptides such as calcitonin gene-related peptide (CGRP). Peptidergic and non-peptidergic neurons express different repertoires of ion channels and receptors involved in signal transduction and innervate distinct peripheral and central targets (Woolf and Ma, 2007). The neuropeptide calcitonin gene–related peptide (CGRP) is conspicuously enriched in the trigeminal ganglion, the region that contains the highest concentration of CGRP–expressing neurons (–50% of the neurons store CGRP) (Uddman et al., 1986) and the cranial dura is well innervated by a dense network of CGRP fibres (Strassman et al., 2004). To study CGRP nociceptive signaling mechanisms I selectively identify CGRP fibres using a fluorescent transgenic CGRP-EGFP mouse (Figure 2.3A and B; Figure 2.4 and 2.5). Non-peptidergic nociceptors possess membrane-associated glycoconjugates that bind the plant lectin Isolectin B4 from *Griffonia simplicifolia* and have been shown in the dura to constitute a fibre population distinct from those containing CGRP (Soyguder, 1999). For the selective identification of non-peptidergic dural nociceptive fibres I briefly perfuse a 25 ml solution containing fluorescently tagged IB4 that brightly labels the membranes of the fibres (Figure 2.6A-C). I combined IB4 labeling with the Ca^{2+} indicator Rhod-2 (Figure 2.6B) for functional imaging studies (see below). All functional imaging experiments (below) were performed on unmyelinated nociceptive fibres. Myelinated fibres were easily identified in the IR–transmitted image (Figure 2.7A) and confirmed with a brief perfusion of a 10 ml solution containing the myelin stain FluoroMyelin Red® (Figure 2.7A – C).

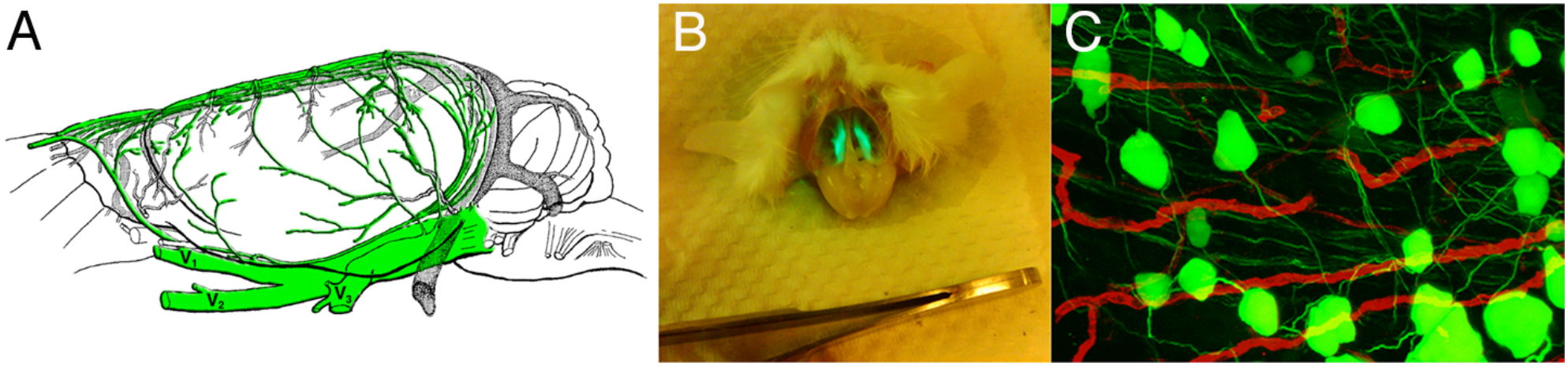

Figure 2.4. Trigeminal ganglion expression of EGFP in the α-CGRP-EGFP mouse.
A) Topography of the dense trigeminal nociceptive innervation of the cranial dura mater is highlighted in green. V1; ophthalmic, V2; maxillary, and V3; mandibular nerves originating from the trigeminal ganglion are labeled. Stippled print labels venous sinuses and venous vessels. **B**) Intense level of EGFP fluorescence labeling in the trigeminal ganglia of the transgenic α-CGRP-EGFP mouse *Tg* (*Calca*-EGFP) FG104 Gsat illuminated by an LED flashlight (*NightSea*) in combination with a custom interference filter to provide effective fluorescence excitation (note the ventral surface of the brain is completely non-fluorescent). **C**) Two-photon image stack through a section of the ophthalmic division of the trigeminal ganglion shows the density of CGRP-EGFP positive cells. Vessels are highlighted in red from an intracardial perfusion with Alexa 594 IB4.

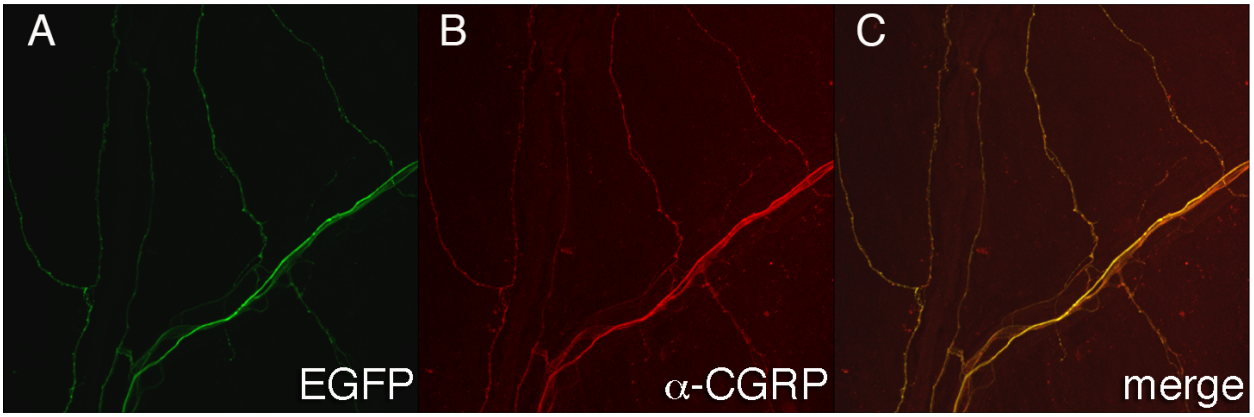

Figure 2.5. Immunohistochemical confirmation of dural α-CGRP-EGFP in the α-CGRP-EGFP transgenic mouse.
A) α-CGRP-EGFP expressing fibres in the dura mater from the transgenic mouse. **B**) The anti α-CGRP antibody (Alexa Fluor 594) confirms the pattern of expression of the α-CGRP-EGRP fibres in the dura mater; merged image (C).

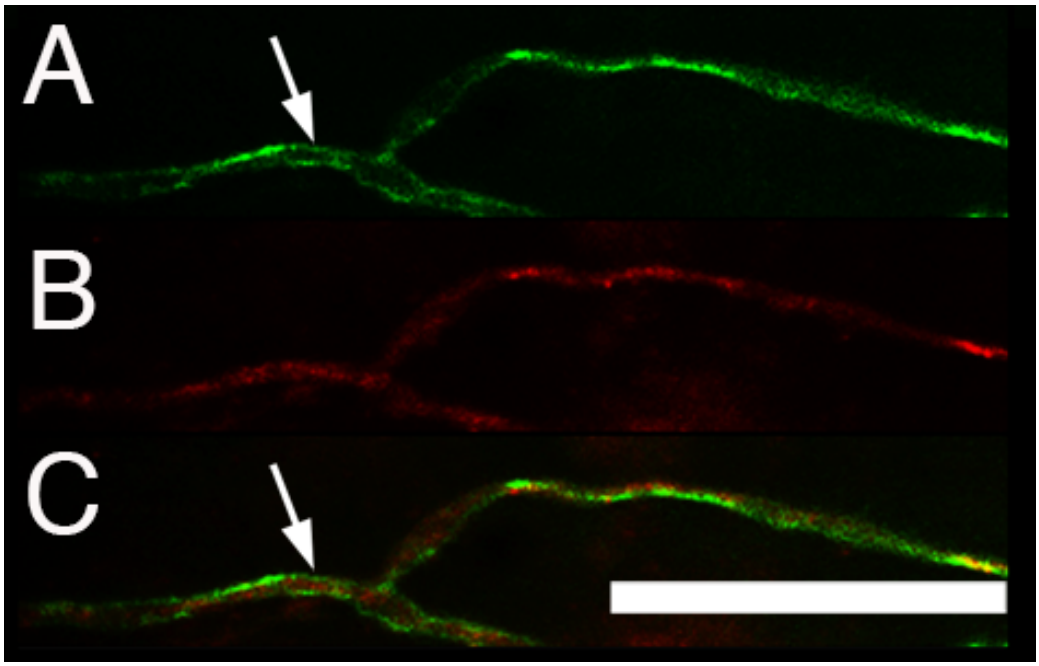

Figure 2.6. Identification of nonpeptidergic fibres in the dura with conjugated IB4. **A**) Image of an isolectin B4-positive nonpeptidergic labeled fibre taken with widefield epifluorescence imaging at high magnification highlights the membrane patterned labeling (arrows) and contrasts the cytosolic labeling of the calcium indicator Rhod-2 (**B**) and merged images (**C**).

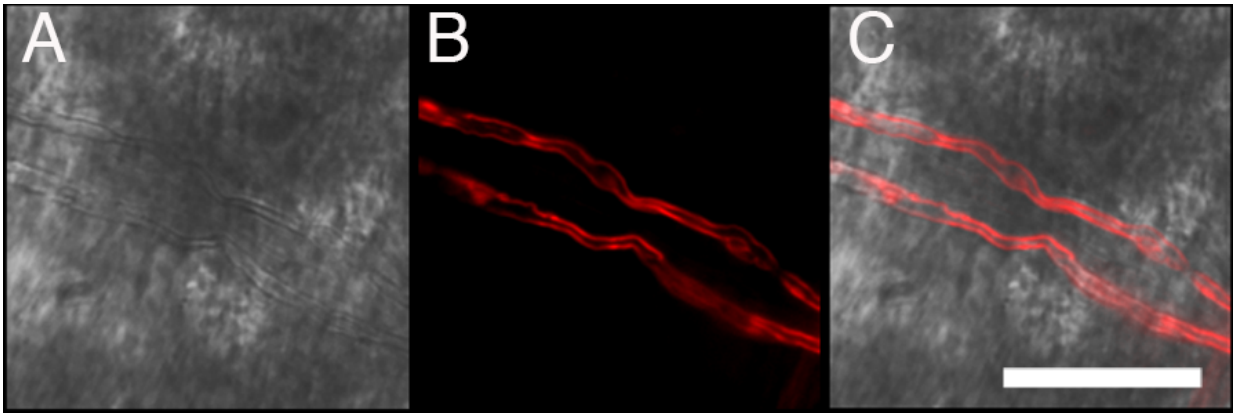

Figure 2.7. Identification of myelinated A-delta fibres in the dura.
A) Myelinated fibres are easily identified with widefield IR–transmitted imaging. FluoroMyelin Red ™ labeled myelin fibres seen using standard epifluorescence and CCD camera detection (**B**) confirms the identical fibres seen with transmitted imaging; merged image (**C**). Scale bar 50 μm.

2.4.3 Functional imaging in pain fibres

Here I demonstrate that high–resolution functional imaging can be performed on individual pain fibres in the dural–skull preparation. In neurons, action potentials trigger large and rapid changes in cytosolic free Ca^{2+} and imaging with fluorescent Ca^{2+} indicators has been widely used to monitor neuronal spiking across populations of CNS neurons. I find that very brief (10 – 20 sec) application of the Ca^{2+} indicator Rhod–2 loads peripheral pain fibres and that single action potential-evoked Ca^{2+} transients with large signal–to–noise ratios may be imaged within individual fibres ($\Delta F/F$ = 19.8 ± 1.2%, 72 fibres in n = 60 preparations) over long experimental periods without decrement ($\Delta F/F$ % control = 100 ± 3.2% at the 80 minute experimental time point, n = 4) (Figure 2.8A and B). We performed a series of experiments to verify that the Ca^{2+} transients were the result of action potentials evoked from the electrical stimulation. Stimulation within 10 μm of the afferent termination sites of the pain fibres evoked 'all–or–none' Ca^{2+} transient responses during experiments of: ***1***) variable stimulus intensity; 140 μA: ($\Delta F/F$ = 100 ± 4.9%) vs no transient observed at 110 μA stimulation: ($\Delta F/F$ = -0.2 ± 0.2%, n = 5), ***2***) TTX added; physiological solution: ($\Delta F/F$ = 100 ± 8.5%) vs no transient observed once TTX was added to the solution, +TTX: ($\Delta F/F$ = -1.1 ± 0.1%, n = 5), and ***3***) zero Ca^{2+}: 2 mM Ca^{2+}: ($\Delta F/F$ = 100 ± 2.5%), vs no transient observed once Ca^{2+} was removed from the solution, 0 mM Ca^{2+}: ($\Delta F/F$ = -1.6 ± 0.2%, n = 5) (Figure 2.9A and B).

Next I show that UV photolysis experiments may be performed both *in* and *at* individual pain fibres. Ca^{2+} transients from the photolysis of the Ca^{2+} cage NP–EGTA imaged at the site of photolysis *in* the fibre ($\Delta F/F$ = 135 ± 21.2%, n = 9), 30 μm away ($\Delta F/F$ = 50.1 ± 8.2%, n = 9), and 60 μm away ($\Delta F/F$ = 4.2 ± 0.8%, n = 9) are shown in (Figure 2.10A *above*; B *left*). The transient receptor potential vanilloid 1 (TRPV1) receptor/channel is a polymodal sensor and

molecular integrator of a wide range of painful stimuli that is activated directly by capsaicin, the natural compound responsible for the sharpness of hot chili peppers (Clapham, 2003; Szallasi et al., 2007). TRPV1 sensitivity to capsaicin is considered a principal pharmacological trait of lightly myelinated Aδ– and unmyelinated C–fibre nociceptors and is a hallmark for identifying primary afferent fibres as nociceptors (Kostyuk and Kostyuk, 2009). Ca^{2+} transients from the photolysis of the novel and selective TRPV1 custom caged molecule BCMACMOC–caged capsaicin (Gilbert et al., 2007) *at* the pain fibre ($\Delta F/F$ = 108 ± 48%) as well as the block by the TRPV1 antagonist capsazepine ($\Delta F/F$ = -0.9 ± 0.2%, n = 8) is shown in (Figure 2.10A *below*; B *right*).

Finally, I show an example of the 'axon reflex' in a CGRP peptidergic pain fibre. The 'axon reflex' theory posits a mechanism whereby both afferent and efferent impulse activity may occur in the same primary afferent sensory neuron at a point of bifurcation: *"...a stimulus applied to one branch of a nerve, which sets up an impulse that moves centrally to the point of division of the nerve, where it is reflected down the other branch..."* (Dorland, 2003). This concept was originally proposed by Bruce 100 years ago (Bruce, 1913) and remains in current textbooks of physiology essentially unchanged from its original description. Figure 2.11 shows an example of the axon reflex in a CGRP peptidergic pain fibre. Stimulation (A) of the fibre termination at location (1) initiated afferent impulse activity that could be seen proximally as a Ca^{2+} transient at location (3), while at the same time efferent impulse activity caused a Ca^{2+} transient in the distal fibre termination at location (2). Similarly, stimulation (B) of the fibre termination at location (2) initiated afferent impulse activity seen proximal as a Ca^{2+} transient at location (3), while at the same time, efferent impulse activity caused a Ca^{2+} transient in the distal fibre termination at location (1). Importantly, both afferent and efferent action potential-evoked

Ca^{2+} transients are of equal amplitude at both distal fibre locations (1 + 2), as indicated by the ratio of transients resulting from stimulations (A + B)

Location 1 $\Delta F/F$(A)/ $\Delta F/F$(B) = 102 ± 9% (n = 5)

Location 2 $\Delta F/F$(A)/ $\Delta F/F$(B) = 98 ± 4% (n = 5)

This physiological phenomenon seems to be well conserved as I found that the axon reflex occurred in all CGRP dural nociceptive fibres I tested for it in (n = 13 preparations).

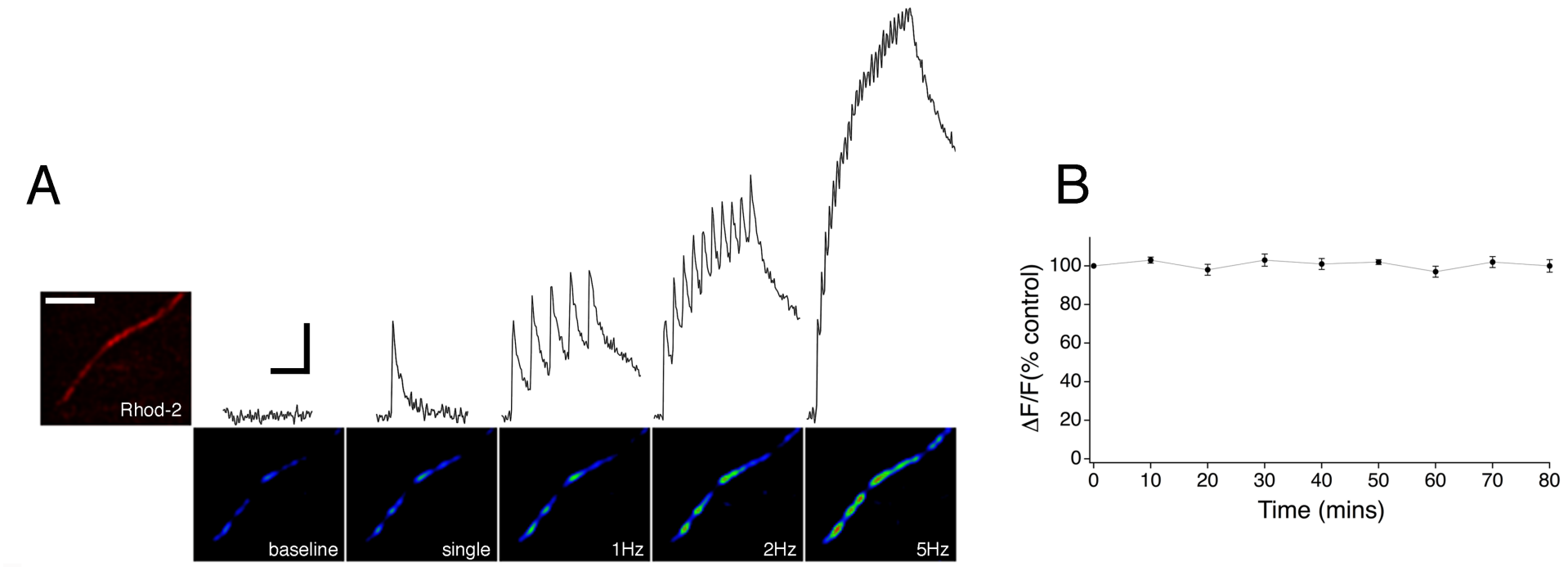

Figure 2.8. Functional imaging in pain fibres.
A) (*leftmost*) Image of a Rhod–2 loaded pain fibre free ending displays the typical region of interest used for the quantification of Ca^{2+} transients. Pseudocolor image series illustrate the fluorescence changes that occurred during the different stimulation frequencies indicated. The corresponding Ca^{2+} transients (*above*) are the average of 5 trials. **B**) Time course of single action potential mediated Ca^{2+} transient responses (n = 4). Scale bars; **A**) 5 μm, 2 s, 10% $\Delta F/F$.

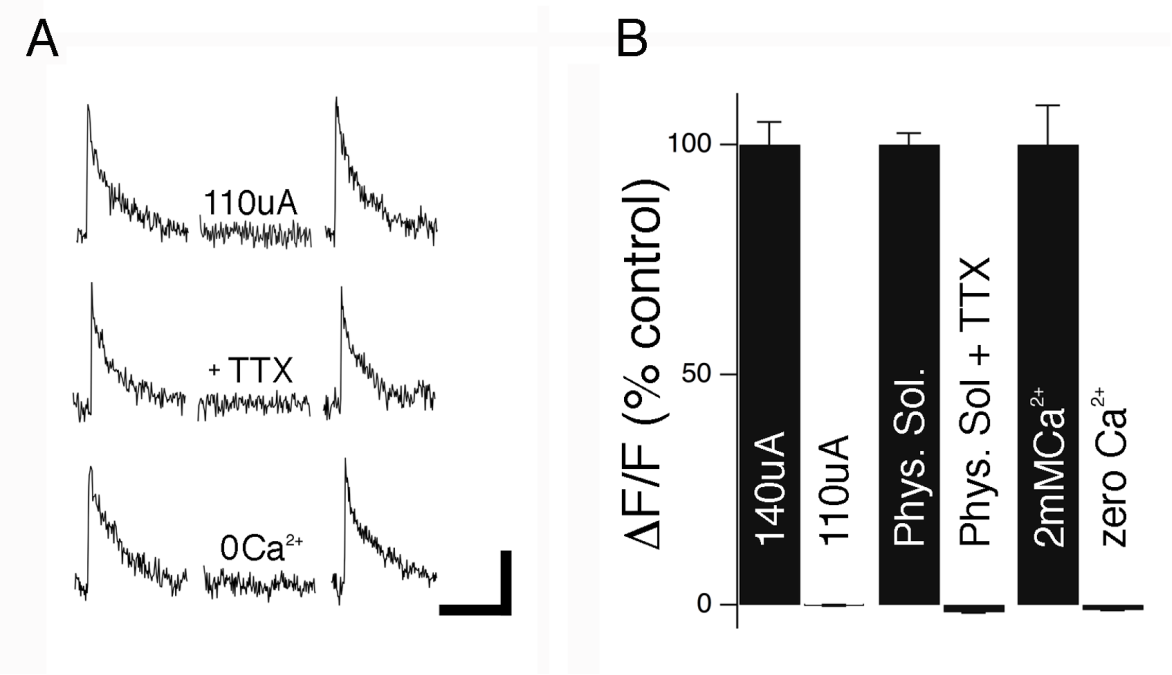

Figure 2.9. Confirmation of action potential-induced Ca^{2+} transients.
A) Electrical stimulation at afferent termination sites of the pain fibres (≤10μm) evoked 'all–or–none' Ca^{2+} transient responses during experiments of; variable stimulus intensity (140 μA or 110 μA) (*upper*), TTX (*middle*), and zero Ca^{2+} (*bottom*) conditions. **B**) Bar graph summaries (Δ*F/F*; % control) of the experimental conditions seen in (**A**); (*n* = 5 for all conditions). Scale bars: 2 s, 10% Δ*F/F*.

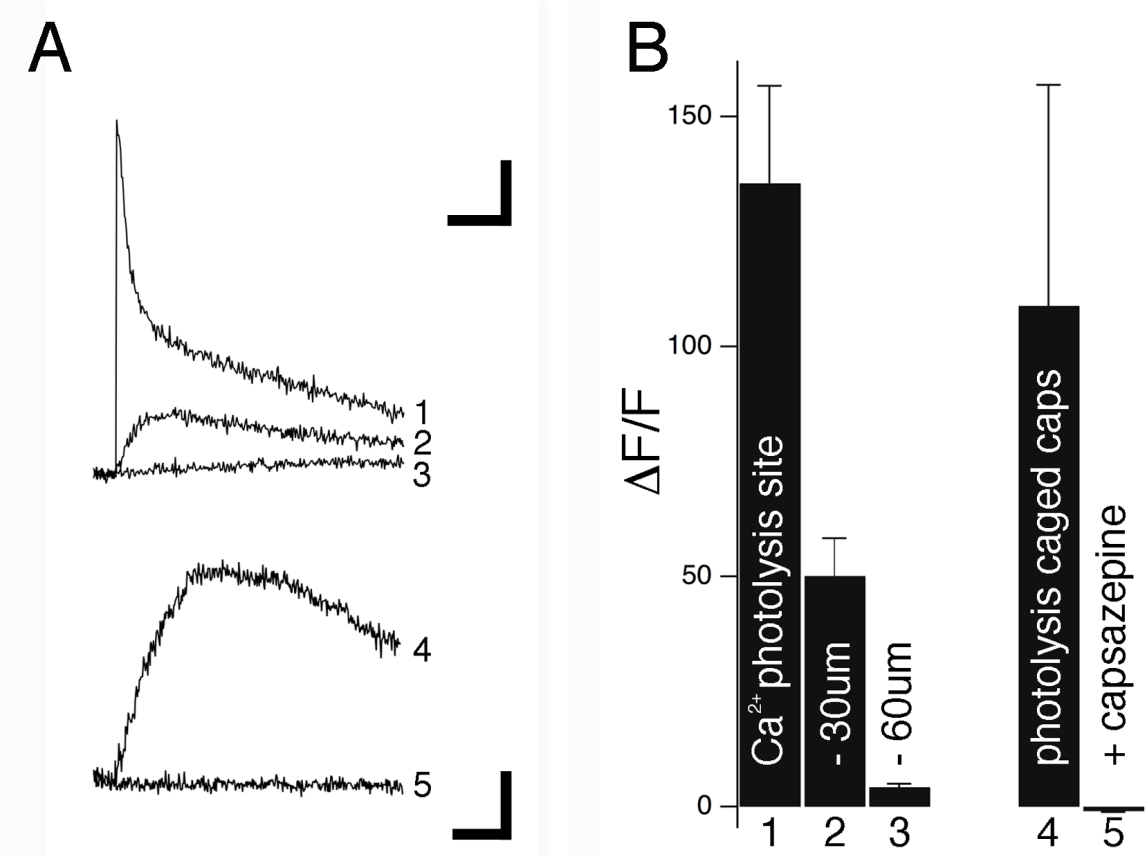

Figure 2.10. Molecular uncaging in and around nociceptive terminals.
A) (*above*) Ca^{2+} transients showing the photolysis of the Ca^{2+} cage NP–EGTA imaged at the site of photolysis on the fibre (1), 30μm away (2), and 60μm away (3). (*below*) Photolysis of the selective TRPV1 custom caged molecule BCMACMOC–caged capsaicin (4) is blocked with the TRPV1 antagonist capsazepine (5). **B**) Bar graph summaries (Δ*F/F*) of the caged NP–EGTA (*n* = 9) and BCMACMOC–caged capsaicin (*n* = 8) photolysis experiments (numbers below bar summaries mark the experimental conditions under which the individual transients seen in (**A**) were acquired). Scale bars: *above* 3 s, 30% Δ*F/F*; *below* 2 s, 40% Δ*F/F*

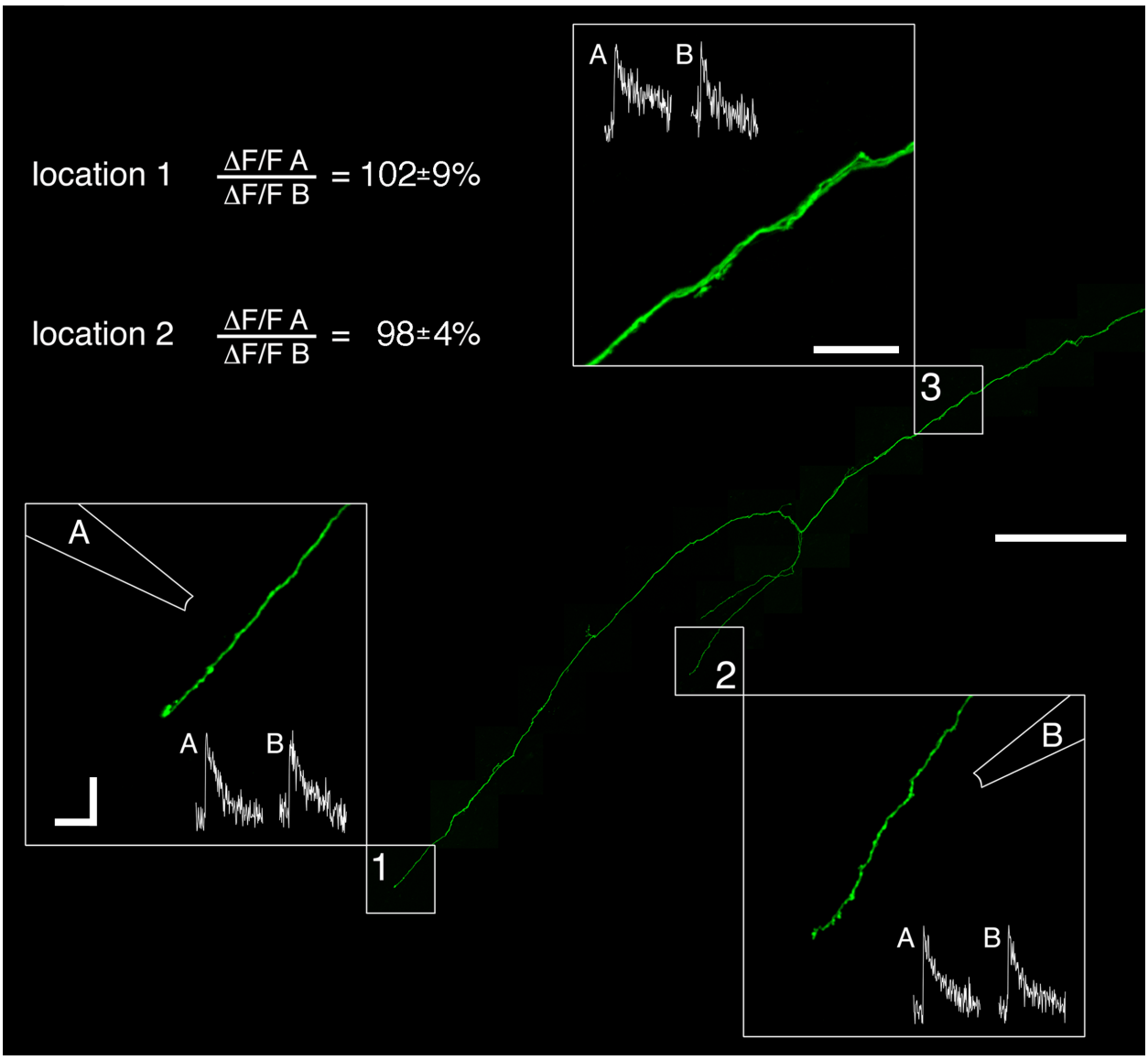

Figure 2.11. The "axon reflex" in dural CGRP fibres.
Image montage shows the peripheral region (>1500μm) of a CGRP–EGFP pain fibre. Two distal terminations (1 + 2) after a bifurcation point as well as a more proximal region (3) are highlighted (white boxes) and at higher magnification to show the locations of the imaged Ca^{2+} transients (A + B). Ca^{2+} transients (A or B) imaged at each location resulted from stimulation of either distal termination location (1 or 2) displayed here as stimulation pipettes marked (A or B) respectively. Evoked Ca^{2+} transients are shown to be of equal amplitude at both distal fibre locations (1 + 2), displayed as the ratio of transients resulting from stimulations (A + B). Ca^{2+} transients were imaged at 25°C. Scale bars: *all transients* 2 s, 10% Δ*F/F*; 25 μm and 200 μm.

2.5 DISCUSSION

I have introduced an experimental imaging approach to directly study physiological functioning within selectively identified nociceptive fibre free nerve endings that terminate in the cranial dura mater. I have developed an *en bloc* mouse dural–skull preparation that preserves individual nociceptive fibre integrity for millimeters. The thinness of the mouse dural layer that is maintained over the expanse of the parietal skull bone offers unprecedented tissue properties for optical microfluorometric imaging in an intact preparation. To my knowledge, this is the first study to show high–resolution functional imaging selectively within individual nociceptive fibre terminations.

Unmyelinated nociceptive fibre terminations have been shown to outnumber myelinated fibres in the parietal dura mater by a ratio of 10:1 (Fricke et al., 2001). Because the small size of the unmyelinated nerve endings makes them inaccessible with conventional electrophysiological techniques I applied widefield epifluorescence microscopy to image action potential–evoked Ca^{2+} signaling as a means for measuring neuronal spiking. I found that single action potential-evoked 'all–or–none' Ca^{2+} transients with large signal–to–noise ratios could be imaged selectively within unmyelinated nociceptive fibre free nerve endings (~1 μm diameter). High–resolution functional imaging could be performed over long experimental periods without decrement, demonstrating the stability of the preparation for examining terminal afferent physiology and pathophysiology.

The nature of the afferent signal depends on a complex interaction between the distribution, density, and biophysical properties of ion channels and molecular transducers present in the afferent terminals, as well as the morphological features of the terminal arbor (Kostyuk and Kostyuk, 2009; Woolf and Ma, 2007). Significant quantitative differences may

exist between the patterns of expression of ion channels and signaling systems in the somata and peripheral processes of the afferent neuron. Yet, the active membrane properties of the afferent neuron soma and its peripheral terminal endings are most commonly considered identical, and the very real possibility of significant quantitative differences has usually been neglected (Kostyuk and Kostyuk, 2009). High–resolution functional optical imaging in the *en bloc* dural–skull preparation now provides an opportunity to begin to study the fundamental physiological processes of activation, transduction, sensitization, and propagation of the nociceptive signal in peripheral pain fibre terminations. This opens up a new window for examining physiological functioning in peripheral nociceptive fibres and to advance our understanding of the peripheral processes involved in pain pathophysiology.

Chapter 3

Sumatriptan inhibition of N-type calcium channel mediated signaling in dural CGRP terminal fibres

3.1 ABSTRACT

The selective 5-HT_1 receptor agonist sumatriptan is an effective therapeutic for migraine pain yet the antimigraine mechanisms of action remain controversial. Pain-responsive fibres containing calcitonin gene-related peptide (CGRP) densely innervating the cranial dura mater are widely believed to be an essential anatomical substrate for the development of migraine pain. $5HT_1$ receptors in the dura colocalize with CGRP fibres and thus provide a possible peripheral site of action for sumatriptan. In the present study, I used high-resolution optical imaging within individual mouse dural CGRP nociceptive fibre terminations and found that application of sumatriptan caused a rapid, reversible concentration-dependent inhibition in the amplitude of single action potential-evoked Ca^{2+} transients. Pre-application of the non-selective 5-HT_1 antagonist GR127935 or the selective 5-HT_{1D} antagonist BRL 15572 prevented inhibition while the selective 5-HT_{1B} antagonist SB 224289 did not, suggesting this effect was mediated selectively through the 5-HT_{1D} receptor subtype. Sumatriptan inhibition of the action potential-evoked Ca^{2+} signaling was mediated selectively through N-type Ca^{2+} channels. Although the T-type Ca^{2+} channel accounted for a greater proportion of the Ca^{2+} signal, it was not affected by the sumatriptan inhibition. My findings support a peripheral site of action for sumatriptan in inhibiting the activity of dural pain fibres selectively through modulation of a single Ca^{2+} channel subtype. This finding adds to our understanding of the mechanisms that underlie the clinical effectiveness of $5HT_1$ receptor agonists such as sumatriptan and may provide insight for the development of novel peripherally targeted therapeutics for mitigating the pain of migraine.

3.2 INTRODUCTION

The serotonin 5-HT_1 receptor agonist sumatriptan and the other triptans are selective and effective therapies for the acute treatment of migraine pain (Bigal et al., 2009; Moskowitz and Cutrer, 1993; Sprenger and Goadsby, 2009; Tfelt-Hansen et al., 2000; Tfelt-Hansen and Koehler, 2011). However, despite the success of the triptans in the clinical setting, the anatomical locus of their antimigraine activity remains unresolved, and both peripheral and central nervous system sites of action are likely (Ahn and Basbaum, 2005; Akerman et al., 2011; Bartsch et al., 2004; Durham and Russo, 2002; Humphrey and Feniuk, 1991; Lambert, 2010; Levy et al., 2004; Mehrotra et al., 2008; Tfelt-Hansen, 2010). Beyond providing insight into migraine neuropathology, understanding the anatomical locus of their action may lead to the development of novel therapeutics to better manage the disorder, and so continues to be an area of intense investigation.

The neuropeptide calcitonin gene-related peptide (CGRP) is hypothesized to play an important role in migraine pathology (Durham, 2008; Ho et al., 2010). Serum levels of CGRP have been shown to be increased during migraine attack (Goadsby et al., 1990), although, this observation has been questioned (Tvedskov et al., 2005), migraine patients infused with CGRP develop a delayed headache, that meets the criteria of a migraine (Lassen et al., 2002), and infusion of CGRP receptor antagonists has been shown to effectively treat migraine pain (Durham and Vause, 2010; Fischer, 2010; Hoffmann and Goadsby, 2011). Importantly, treatment of migraine headache pain with sumatriptan has been shown to correlate with normalization of CGRP levels –an effect that paralleled migraine resolution (Edvinsson and Ho, 2010; Goadsby and Edvinsson, 1993; Sarchielli et al., 2006; Stepien et al., 2003).

CGRP fibres that originate from the trigeminal ganglion densely innervate the

intracranial meninges, in particular the cranial dura mater (Messlinger et al., 1993; Strassman et al., 2004) and this innervation is hypothesized to be the essential anatomical substrate for the development of migraine pain (Messlinger, 2009; Olesen et al., 2009; Pietrobon and Striessnig, 2003). 5-HT_1 receptors colocalize with CGRP immunoreactive fibres in the cranial dura and the antimigraine actions of sumatriptan may involve the inhibition of CGRP release from these terminals (Hargreaves, 2007; Harriott and Gold, 2008; Ho et al., 2010; Longmore et al., 1997). Consistent with this proposal, elevated CGRP levels after stimulation of the trigeminal ganglion are normalized with sumatriptan treatment both in animal models and in humans (Buzzi et al., 1991; Goadsby and Edvinsson, 1993; Limmroth et al., 2001) and in primary cultures from rat trigeminal ganglia, CGRP secretion under conditions simulating migraine pathology is inhibited by sumatriptan (Durham and Russo, 1999).

I have recently introduced a new experimental approach that, for the first time, allows examination of the peripheral signaling processes selectively within individual CGRP terminal pain fibres in the dura (Baillie et al., 2011). In the present study, I use high-resolution optical imaging to directly test the hypothesis that 5-HT_1 receptor activation by sumatriptan has a peripheral site of action by inhibiting action potential mediated Ca^{2+} signaling in terminals of CGRP pain fibres.

3.3 MATERIALS AND METHODS

3.3.1 Dissection

This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use. 41 Transgenic mice *Tg* (*Calca*-EGFP) (*GENSAT Project at Rockefeller University*) (1 month-adult) were dissected as previously described (Baillie et al., 2011). Briefly, the head from an anaesthetized animal was removed from the body at the atlanto–occipital joint. The skin was cut along the skull sagittally and gently moved laterally on both sides. Dissection scissors were then inserted into the foramen magnum and the skull with brain was cut laterally below the occipital and parietal bones on both sides. The two lateral cuts were joined sagitally at the ventral section of the frontal bone. The remaining skull and brain was placed in a bath of physiological solution and the brain carefully removed from the skull leaving intact dura mater and arachnoid mater layers attached to the skull. The interparietal bone was removed by cutting along the lambdoidal suture and the frontal bone removed by cutting along the coronal suture. Two separate and complete parietal bone-dural layer preparations were obtained with a remaining cut along the sagittal suture. Individual dural-skull preparations were placed dural layer up in a microscope chamber and continuously perfused with physiological saline consisting of (in mM): 125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 10 Glucose, 2 $MgCl_2$, 1.25 NaH_2PO_4, and 2 $CaCl_2$.

3.3.2 Calcium indicator application

All functional imaging experiments were performed on unmyelinated nociceptive fibres (Baillie et al., 2011). Afferent nociceptive fibres were selectively loaded with the membrane permeant high affinity Ca^{2+} indicator Rhod-2 AM (*Biotium*) by applying a small piece of indicator solution soaked Surgifoam® to the rostral area of the preparation for 10 - 20 s. The

220 μM indicator solution was made up fresh each day in physiological solution containing 5% Pluronic F-127/DMSO.

3.3.3 Immunohistochemistry

Dural skull preparations were dissected as above and placed in 10% formalin for 15 minutes. Preparations were then rinsed in 0.01 M phosphate buffered saline (PBS) 3 times for 10 minutes each before being placed in a blocking solution consisting of 10% goat serum, 1% bovine serum albumin (BSA) and 0.01 M PBS containing 0.3% triton for 60 minutes. A custom primary antibody for the 5-HT_{1D} receptor (Potrebic et al., 2003) was diluted to 1:60000 with 5% goat serum, 1% BSA, and 0.01 M PBS containing 0.3% triton and preparations added to this mixture and incubated at room temperature for 48 hours. Preparations were then rinsed in 0.01 M PBS containing 1% goat serum 3 times for 10 minutes each. The secondary antibody, Alexa Fluor 594 goat anti-rabbit IgG (*Invitrogen*) was diluted to 1:500 with 0.01 M PBS containing 1% goat serum and the preparations incubated at room temperature for 1 h. The preparations were then rinsed 3 times for 10 minutes each before imaging.

3.3.4 Widefield epifluorescence microscopy

I used the X-Cite 120PC system (*EXFO Electro-Optical Engineering Inc.*) light source (12% intensity) directly coupled to an Olympus BX51WIF upright research microscope by a 3 m liquid light guide. The light intensity was attenuated with two neutral density filters in series (Olympus N.D.25 and N.D.6) and shutter controlled with a ProScan II Controller (*Prior Scientific*). The illumination and fluorescence light was filtered using a TRITC filter set, a FITC filter set, or a Texas Red filter set. Epifluorescence was detected with two high sensitivity cooled CCD cameras. The 16-bit, 512 × 512 ImagEM EM CCD Camera (C9100-13,

Hamamatsu) cooled to -65 °C and the 16-bit, 1344 × 1024 ORCA-R2 CCD camera (C10600-10B, *Hamamatsu*) cooled to -35 °C.

3.3.5 Analysis of calcium signals

Images were acquired at 20 frames/s using 2 × 2 binning and a minimum number of total images taken to reduce photodynamic damage. Increasing fluorescence baseline, steadily diminishing transients, and/or changes in fibre morphology were considered indicative of photodynamic damage, and fibres showing these changes were discarded. Fluorescence signals were converted to relative fluorescence changes over time and expressed in percentages, defined as $\Delta F/F = ((F_1 - B_1) - (F_0 - B_0))/(F_0 - B_0)$, where F_1 and F_0 are fluorescence in the terminal fibre at any given time point and at the beginning of the experiment, respectively, and B_1 and B_0 are the background fluorescence at any given time point and at the beginning of the experiment, respectively. Background values were taken from an adjacent area located at least 10 μm from imaged areas. To quantify the magnitude of fluorescence change, the peak amplitude of the transient was measured. During drug application, responses were considered stable if < 5% variability was observed over the ~ 10 min control period and baseline fluorescence did not change. The average magnitude of the Ca^{2+} transients before drug application was set as 100%, and the average of four Ca^{2+} transients in stable drug and wash conditions normalized to predrug conditions was taken as the magnitude of drug effect and drug recovery, respectively. Results are shown as means ± s.e.m. Statistical analysis was done using an independent group *t* - test (two-tailed); significance was achieved when $P < 0.05$.

3.3.6 Electrical stimulation

A Master 8 - CP, software controlled 8-channel pulse stimulator and ISO-Flex stimulus isolator unit (*A.M.P.I.*) were used to deliver electrical stimulations via 1 μm bipolar tungsten

electrodes (*WPI*). Stimulation intensity was kept just above threshold to elicit action potentials (140 - 180 μA for a duration of 100 μs) and was not adjusted throughout the course of an experiment.

3.3.7 Drugs

Sumatriptan (500 nM - 40 μM) (*Sigma*), GR 127935 (300 nM) (*Tocris*), BRL 15572 (10 nM) (*Tocris*), SB 224289 (10 nM) (*Tocris*), nifedipine (10 μM) (*Sigma*), ω-conotoxin GVIA (1 μM) (*Alomone labs*), ω-agatoxin IVA (200 nM) (*Alomone labs*), and NNC 55-0396 dihydrochloride (20 μM) (*Tocris*) were all bath applied.

3.4 RESULTS

3.4.1 Sumatriptan inhibition of action potential evoked Ca^{2+} transient amplitude.

To study Ca^{2+} signaling in the terminals of CGRP-containing nociceptive fibres I selectively identified individual fibres using a fluorescent transgenic CGRP-EGFP mouse (Baillie et al., 2011). Single action potential mediated Ca^{2+} transients were evoked by electrical stimulation (single pulse, 140 - 180 μA; 100 μs) at distances greater than 500 μm proximal to the distal fibre terminations (Figure. 3.1). I found that bath application of sumatriptan caused a concentration-dependent inhibition in the amplitude of the evoked Ca^{2+} transient (500 nM – 40 μM; n=5 for each concentration) (Figure. 3.2). The lowest concentration of sumatriptan to achieve maximal inhibition (20 μM) was used in all subsequent experiments. I used a custom primary antibody for the 5-HT_{1D} receptor (Potrebic et al., 2003) because the serotonin 5-HT_{1D} receptor subtype has been shown to be selectively expressed in primary afferent neurons and not in peripheral tissues (as the 5-HT_{1B} receptor) (Longmore et al., 1997), and found punctate $5HT_{1D}$ immunoreactive labeling in the CGRP terminating nociceptive fibres (Figure. 3.3). I performed Ca^{2+} transient amplitude timecourse experiments and found that bath application of sumatriptan caused a rapid reversible inhibition in the amplitude of the Ca^{2+} transient (40.8 ± 1.4%; n=7) (Figures. 3.4 + 3.5). Below the time course traces are examples of action potential-evoked Ca^{2+} transients before, during and after washout of sumatriptan. Sumatriptan-mediated inhibition in the amplitude of the Ca^{2+} transient was prevented by pre-application of the 5-HT_1 antagonist GR127935 (300 nM) or the selective 5-HT_{1D} antagonist BRL 15572 (10 nM); Ca^{2+} transients remained at 102.7 ± 3.3%; n=5 and 99.7 ± 1.4%; n=5 of control conditions respectively (Figure. 3.4+3.5). Pre-application of the selective 5-HT_{1B} antagonist SB 224289 (10 nM) did not block the sumatriptan-mediated inhibition in the amplitude of the Ca^{2+} transient; Ca^{2+} transients

decreased to 63.7 ± 1.0%; n=5 of control conditions (Figures. 3.4 + 3.5). It should be noted that sumatriptan does not affect the baseline (un-evoked) Ca^{2+} signal in terminal CGRP fibres (98.9 ± 0.5% of control; n=6) in contrast to studies performed in primary cultures of trigeminal neurons, where a large sustained Ca^{2+} influx was noted (Durham and Russo, 1999, 2003).

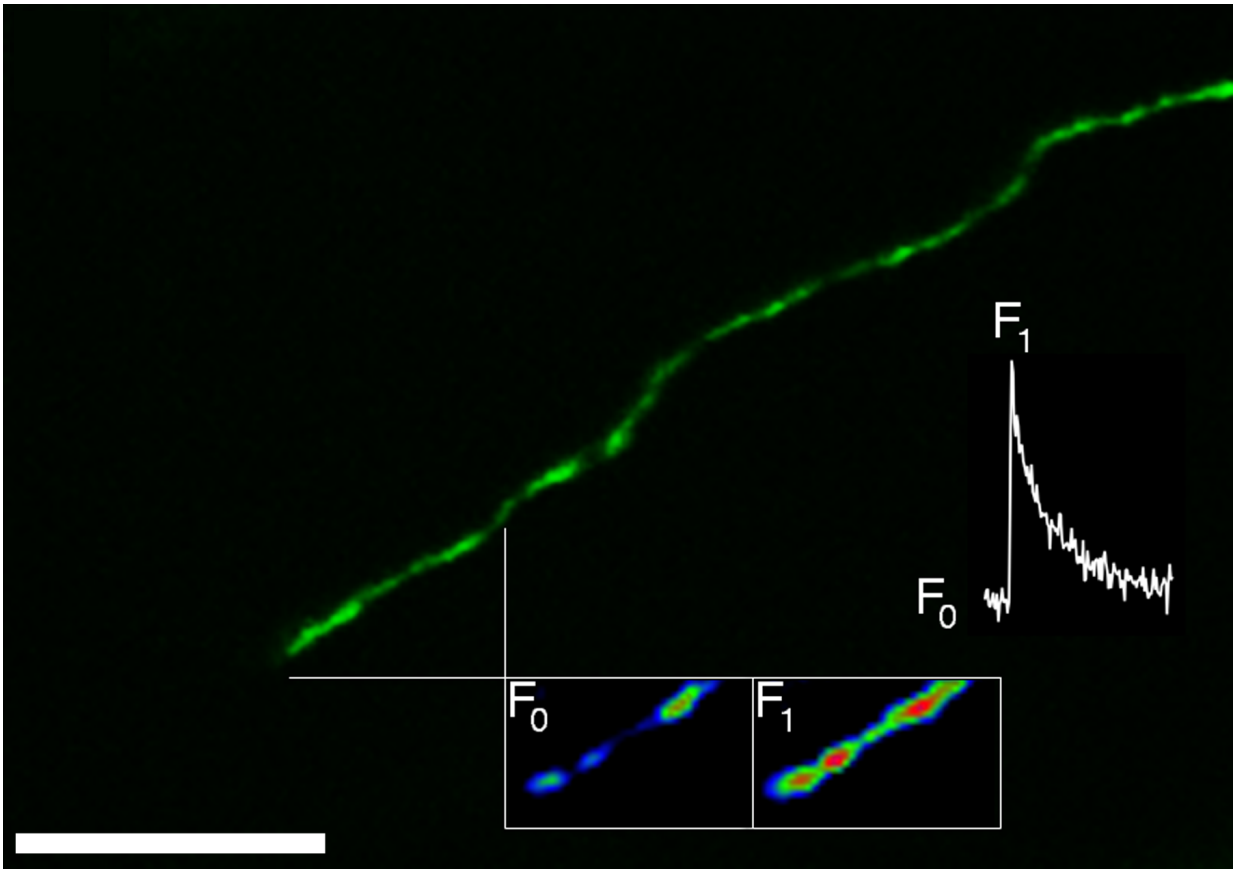

Figure 3.1. A typical imaging region and action potential-evoked fluorescent Ca^{2+} response in a CGRP-EGFP terminating nociceptive fibre.
A CGRP-EGFP terminating nociceptive fibre showing a typical region from which fluorescent transients were acquired and quantified, along with a pseudocolour inset of baseline Rhod-2 fluorescence (F_0), and a single action potential-evoked signal (F_1) with the corresponding transient displayed above. Scale bar: 20 μm.

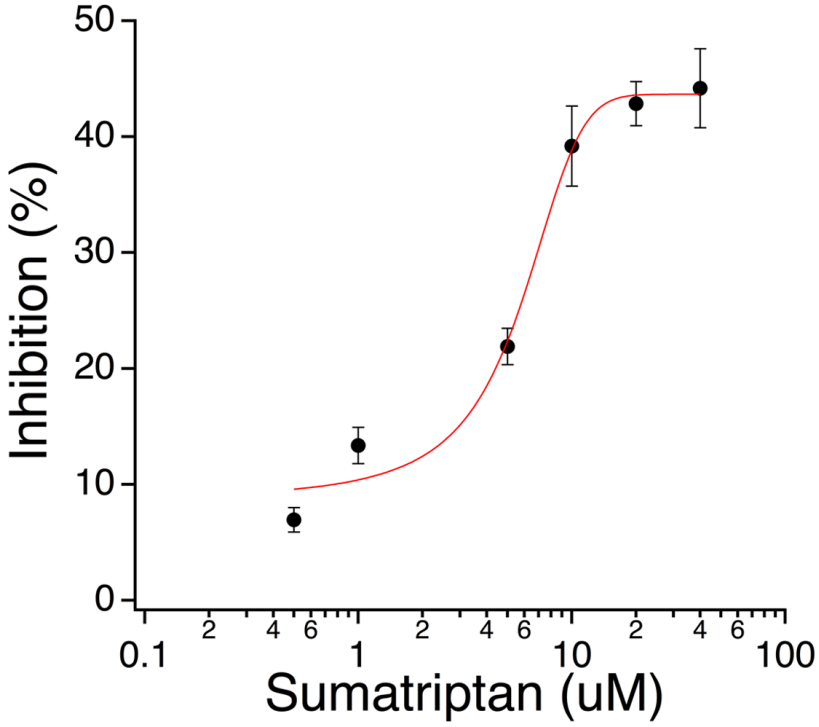

Figure 3.2. Sumatriptan causes a dose-dependent decrease in action potential-evoked Ca^{2+} transient amplitude.
The sumatriptan dose-response curve shows that the amplitude of the Ca^{2+} transient is inhibited with increasing concentrations of sumatriptan.

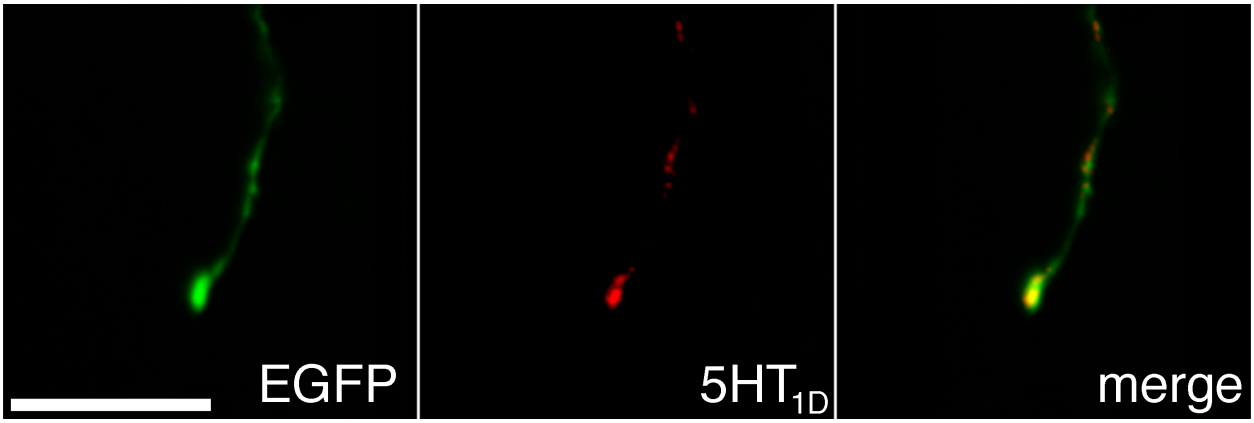

Figure 3.3. Immunohistochemical confirmation of $5HT_{1D}$ receptor expression in dural CGRP fibres.
$5\text{-}HT_{1D}$ receptors are co-localized with terminating CGRP nociceptors. (*Left*) CGRP-EGFP terminating nociceptive fibre shows punctate terminal immunoreactive labeling with a $5\text{-}HT_{1D}$ antibody (*middle and merge*). Scale bar: 20 μm.

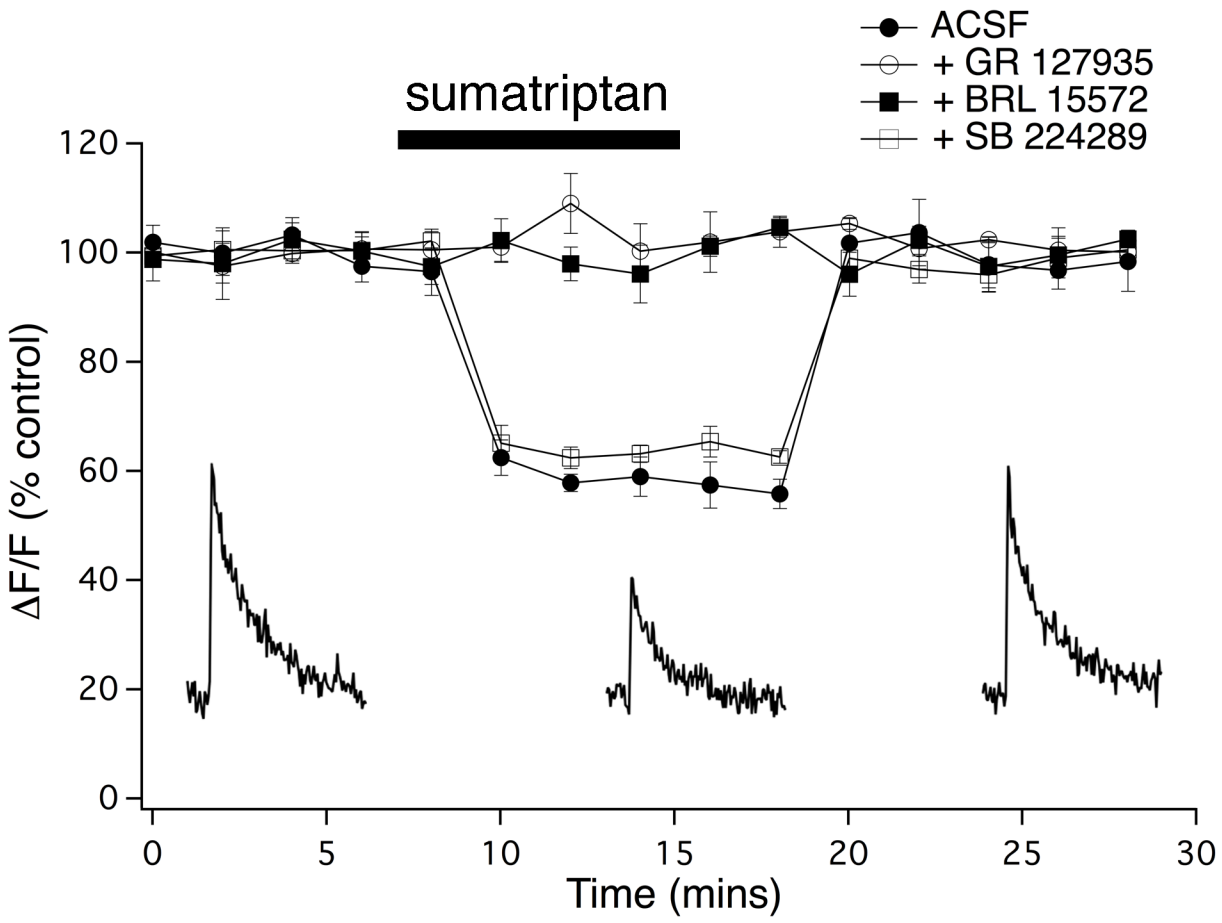

Figure 3.4. Sumatriptan causes a rapid, reversible decrease in action potential-evoked Ca^{2+} transient amplitude via activation of $5HT_{1D}$ receptors.
Graph of Ca^{2+} transient amplitude timecourse experiments showing the effect of sumatriptan application in control conditions (*solid circles*), in the presence of the selective 5-HT_1 receptor antagonist GR127935 (*hollow circles*), the selective 5-HT_{1D} antagonist BRL 15572 (*solid squares*), and the selective 5-HT_{1B} antagonist SB 224289 (*hollow squares*). Individual example transients during control, sumatriptan, and wash conditions taken at the relative timepoints on the graph are shown below.

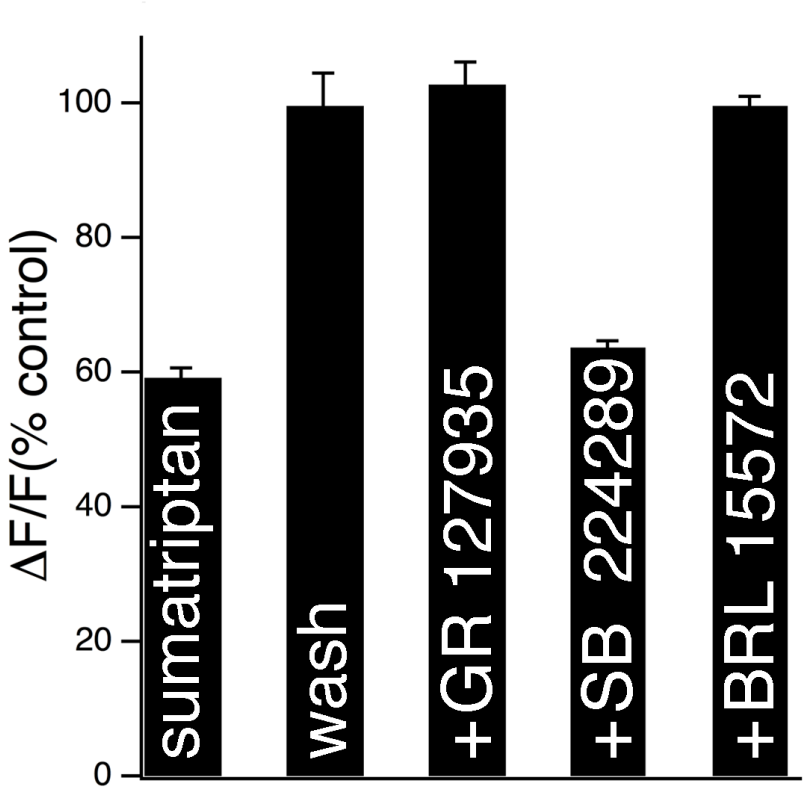

Figure 3.5. Sumatriptan-mediated reduction in Ca^{2+} transient amplitude occurs via $5HT_{1D}$ receptors.
Bar graph summary showing that the sumatriptan reduction in the amplitude of Ca^{2+} transients was completely reversible with wash, blocked by the 5-$HT_{1B/D}$ antagonist GR 127935 and the 5-HT_{1D} antagonist BRL 15572, and was not by the 5-HT_{1B} antagonist SB 224289.

3.4.2 Sumatriptan inhibition of N-type Ca^{2+} channel mediated signaling

I have previously shown that action potential-evoked Ca^{2+} signaling in the terminals of CGRP-containing nociceptive fibres was dependent on extracellular Ca^{2+} suggesting that it depends on an influx through Ca^{2+}channels (Baillie et al., 2011). To examine the inhibitory Ca^{2+} signaling action of $5HT_1$ receptor activation by sumatriptan I first performed a series of selective Ca^{2+} channel block experiments to determine which Ca^{2+} channels mediate the action potential evoked Ca^{2+} signaling. I found that block of P/Q-type Ca^{2+} channels by ω-agatoxin IVA (200 nM) and block of L-type Ca^{2+} channels by nifedipine (10 μM) had no effect on action potential evoked Ca^{2+} transients (101.1 ± 1.5%; n=7 and 100.5 ± 1.4%; n=7 respectively; Figure 3.6A, B + 3.9). The low voltage activated T-type Ca^{2+} channel is known to exist in high density in nociceptors and is likely involved in central and peripheral nociceptive processing (Todorovic and Jevtovic-Todorovic, 2006, 2011). I found that application of the selective T-type Ca^{2+} channel antagonist NNC 55-0396 (20 μM) caused a large irreversible decrease in the amplitude of the Ca^{2+} transient (48.8 ± 1.2%; n=6, Figures. 3.7 + 3.9). Application of sumatriptan in the presence of the T-type Ca^{2+} channel antagonist caused a further decrease in amplitude (40.1 ± 0.9%; n=6, Figures. 3.7 + 3.9), similar to the reduction with sumatriptan in control conditions (40.8 ± 1.4; n=7; Figures. 3.4 + 3.5), suggesting that the T-type Ca^{2+} channel does not mediate sumatriptan inhibition (Figures. 3.7 + 3.9). A large body of work suggests that the N-type Ca^{2+} channel controls neurotransmitter release from peripheral sensory neurons (Altier et al., 2007; Snutch, 2005). I found that brief application of the selective N-type Ca^{2+} channel antagonist ω-conotoxin GVIA (1 μM) caused a large irreversible reduction in Ca^{2+} transient amplitude (40.7 ± 1.0%; n=6; Figures 3.8 + 3.9). Application of sumatriptan in the presence of the N-type Ca^{2+} channel antagonist did not cause a further decrease in the amplitude of the Ca^{2+} transient. This

suggests that sumatriptan inhibition of Ca^{2+} signaling in the terminals of CGRP-containing nociceptive fibres occurs through inhibition of N-type Ca^{2+} channels.

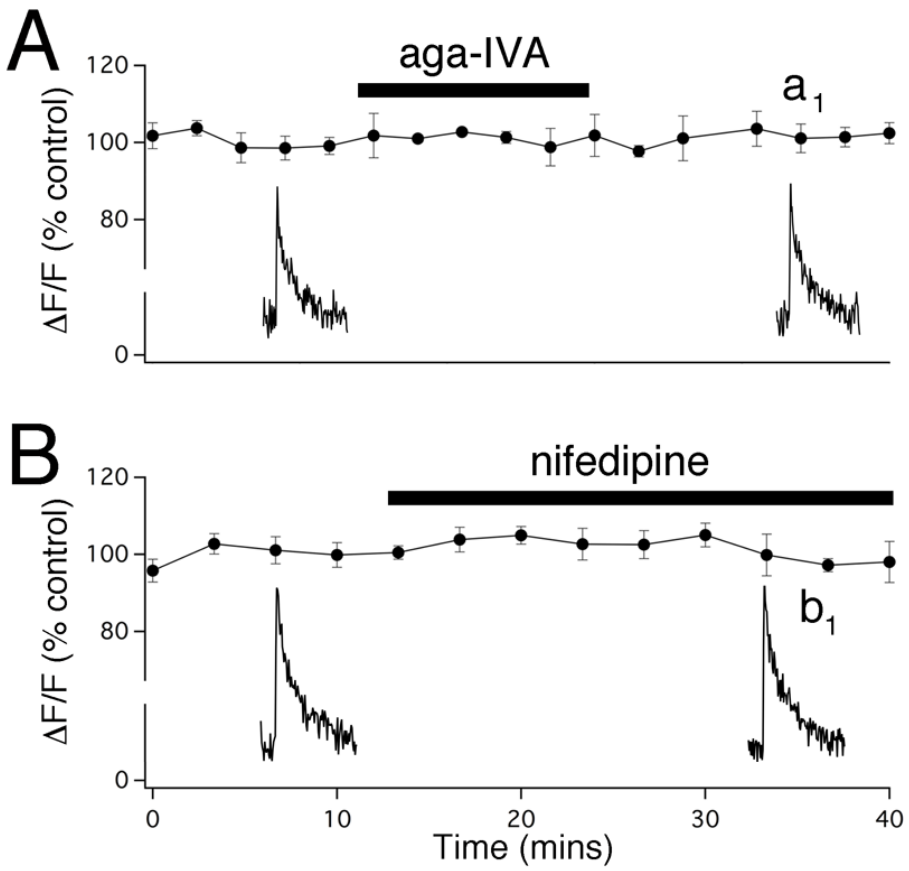

Figure 3.6. Action potential-evoked Ca^{2+} transients do not activate P/Q or L-type voltage gated Ca^{2+} channels in dural CGRP fibres.
Graphs of timecourse experiments showing the effects of Ca^{2+} channel blockers on the single action potential evoked Ca^{2+} transient amplitudes. (A + B) Neither the P/Q Ca^{2+}channel blocker agatoxin-IVA (200 nm) nor the L-type Ca^{2+} channel blocker nifedipine (10 μM) decreased the amplitude of the Ca^{2+} transient.

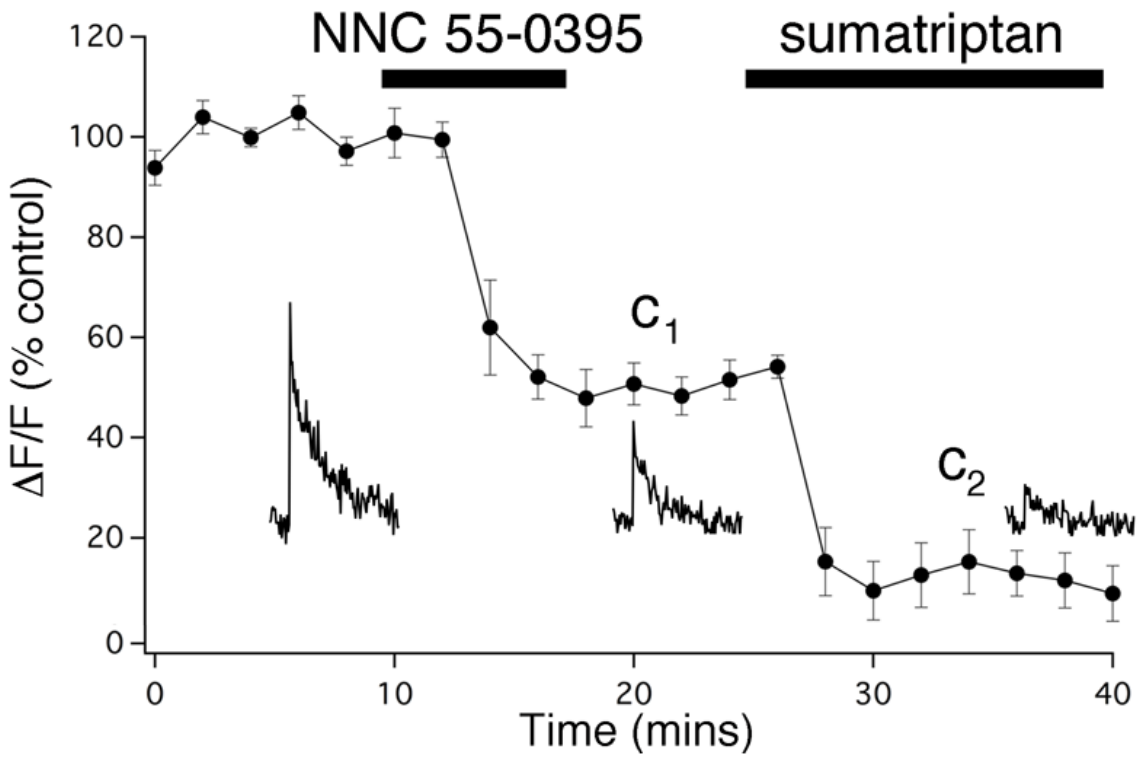

Figure 3.7. The T-type Ca^{2+} channel makes up a significant portion of action potential-evoked Ca^{2+} transients.
The T-type Ca^{2+} channel blocker NNC 55-0936 (20 μM) significantly inhibited the Ca^{2+} transient amplitude. The amplitude of the T-type Ca^{2+} channel mediated inhibition was further reduced by the application of sumatriptan implying that sumatriptan inhibition of Ca^{2+} transient amplitude is not through T-type Ca^{2+} channels.

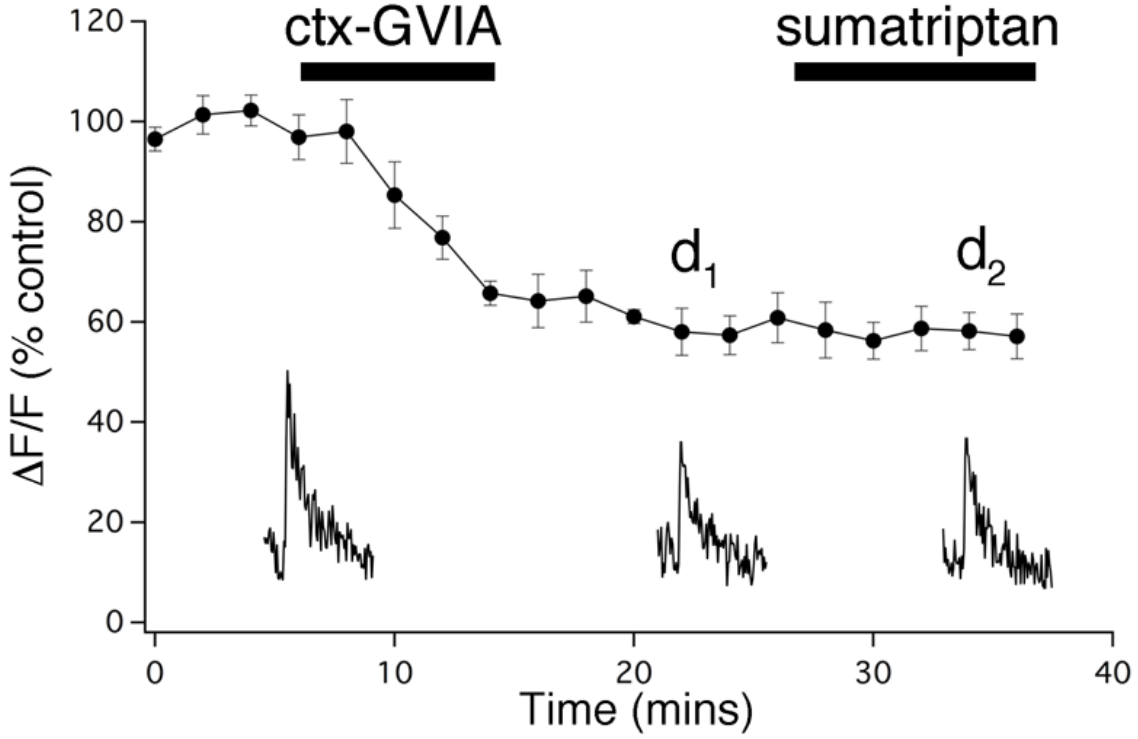

Figure 3.8. N-type Ca^{2+} channels mediate Sumatriptan inhibition of Ca^{2+} signaling. The N-type Ca^{2+} channel blocker conotoxin-GVIA (1μM) significantly inhibited the Ca^{2+} transient amplitude and the amplitude of the N-type Ca^{2+} channel mediated inhibition remained unaffected with sumatriptan application implying sumatriptan inhibition of Ca^{2+} transient amplitude occurs through N-type Ca^{2+} channels.

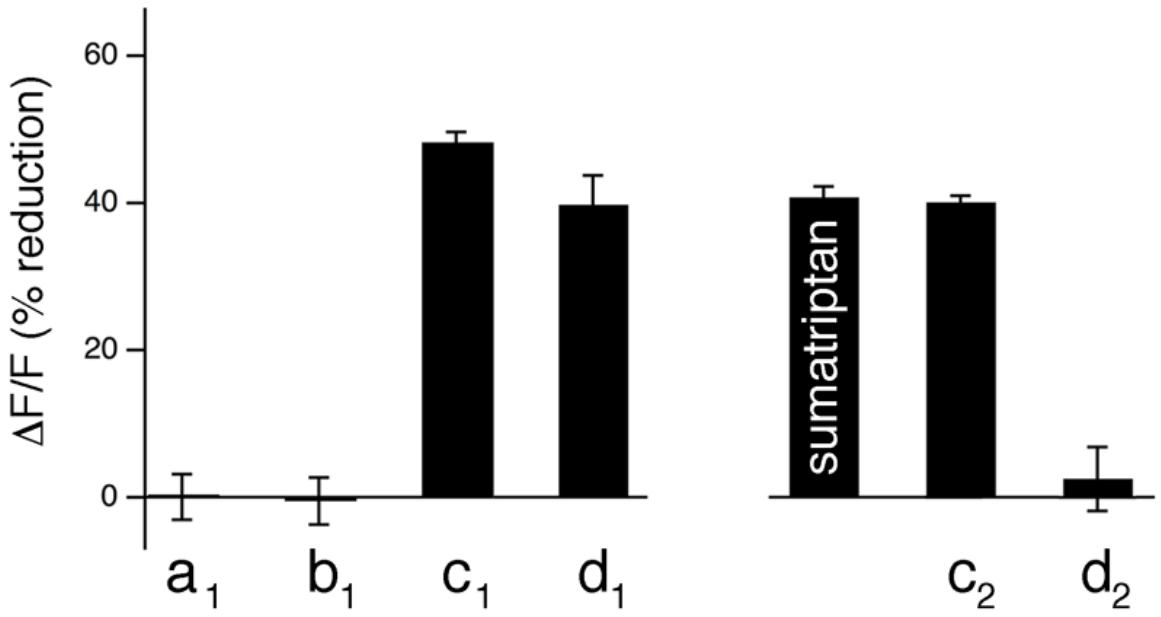

Figure 3.9. Bar graph summaries showing Ca^{2+} transient inhibition by various Ca^{2+} channel blockers and sumatriptan.
Bar graph summaries under the experimental conditions seen in (Figs. 3.6-3.8) showing the inhibition of Ca^{2+} transient amplitudes (ΔF/F; % reduction) at the timepoints shown on the graphs (a_1 - d_1). The magnitude of the sumatriptan-mediated inhibition alone was found to be equal to the magnitude of the sumatriptan mediated inhibition in the presence of the T-type Ca^{2+} channel blocker (c_2), while in the presence of the N-type Ca^{2+} channel blocker, the sumatriptan mediated inhibition was completely occluded (d_2).

3.5 DISCUSSION

In the present study I performed high-resolution functional imaging of Ca^{2+} selectively within individual dural CGRP nociceptive fibre terminations and found that sumatriptan has a peripheral site of action mediated through N-type Ca^{2+} channels to inhibit action potential-evoked Ca^{2+} signaling. CGRP is hypothesized to play an important role in migraine pathology (Ho et al., 2010). My finding that sumatriptan causes selective inhibition of N-type Ca^{2+} channel-mediated Ca^{2+} signaling in dural CGRP fibres is consistent with this hypothesis. Though I do not have a measure of CGRP release from the terminals, it can be inferred that given the significant contribution of the N-type Ca^{2+} channel to the Ca^{2+} signal (~40%), selective inhibition through 5-HT_1 receptor activation should dramatically inhibit neurotransmitter release, since release shows a non-linear dependence on Ca^{2+} influx through voltage dependent Ca^{2+} channels (Bollmann and Sakmann, 2005; Dodge and Rahamimoff, 1967; Katz and Miledi, 1965; Schneggenburger and Neher, 2005). N-type Ca^{2+} channels are also heavily clustered at synaptic sites associated with exocytotic proteins and a large body of work suggests that this channel controls functional coupling between action potentials and evoked neurotransmitter release from nociceptive terminals (Altier et al., 2007; Altier and Zamponi, 2004; Snutch, 2005; Zamponi et al., 2009). It is also well established that N-type Ca^{2+} channels are strongly regulated by G-protein coupled receptors that allow precise control of neurotransmitter release through inhibition of the N-type channel (Currie, 2010; Weiss, 2009).

To the best of my knowledge, this is the first study to show that the activation of 5-HT_1 receptors in trigeminal neurons causes inhibition of Ca^{2+} signaling selectively through a single Ca^{2+} channel subtype, the N-type. The N-type Ca^{2+} channel subtype in particular over other subtypes has been shown to be an attractive target for therapeutic intervention concerning

chronic and neuropathic pain conditions (Snutch, 2005; Zamponi et al., 2009). Indeed, the highly selective N-type Ca^{2+} channel peptide antagonist ziconotide (Prialt®) is a non-opioid analgesic that has recently been used clinically for the amelioration of severe and chronic pain even in morphine-unresponsive situations and without the serious opioid-related side effects such as respiratory depression, addiction, and tolerance (Snutch, 2005). The results presented in the current study suggest that the development of novel N-type Ca^{2+} channel-selective therapeutics may have implications for the treatment of migraine pain. A major advantage for developing compounds that selectively target the dural N-type Ca^{2+} channel and regulate neurotransmitter release is that the medication would not have to cross the blood brain barrier for therapeutic action, thus eliminating serious central nervous system side effects.

I found in dural CGRP fibre terminations that the T-type Ca^{2+} channel accounted for a greater proportion of the Ca^{2+} signal (~50%) than the N-type channel but remained unaffected by the sumatriptan inhibition. Application of sumatriptan in the presence of the T-type Ca^{2+} channel antagonist caused a decrease in the amplitude of the Ca^{2+} signal to the same extent as the reduction seen with sumatriptan in control conditions (~40%). Although T-type Ca^{2+} channels are thought to play a central role in peripheral pain processing, where they are believed to regulate subthreshold excitability in the peripheral nociceptive fibres, they are not thought to control neurotransmitter release (Iftinca and Zamponi, 2009; Jevtovic-Todorovic and Todorovic, 2006; Todorovic and Jevtovic-Todorovic, 2006, 2007, 2011). Consistent with this, it was recently shown that T-type Ca^{2+} channels did not contribute to high potassium-induced CGRP release measured by an enzyme-linked immunoassay in an *ex vivo* rat dural-skull preparation similar to the mouse preparation used in my study (Amrutkar et al., 2011). It is again interesting to speculate on the development of novel antimigraine therapeutics. Compounds that selectively

target the T-type Ca^{2+} channel could be specific enough to ameliorate the pathophysiological activation and/or sensitization mechanisms of the dural nociceptive fibres that are thought to occur during migraine, without modifying neurotransmitter release mechanisms that may have remained unimpaired during the migraine event.

I found that block of P/Q- and L-type Ca^{2+} channels had no effect on action potential evoked Ca^{2+} transients in dural CGRP nociceptive fibre terminations. These findings contrast with those of Amrutkar *et al*. (2011), who found that block of P/Q- and L-type Ca^{2+} channel subtypes each decreased 60 mM potassium-induced CGRP release (Amrutkar et al., 2011). The differences between the two studies may be due to the different stimulation protocols and/or to the different concentrations of the Ca^{2+} channel antagonists used (Sidach and Mintz, 2000).

3.6 CONCLUSION

The antimigraine activity of the 5-HT_1 receptor agonist sumatriptan is well established, yet the anatomical locus and mechanisms of action remain unresolved. In the present study I have shown that sumatriptan acts peripherally at individual dural CGRP nociceptive fibre terminations to inhibit action potential-evoked Ca^{2+} signaling selectively through N-type Ca^{2+} channels. This finding provides insight for the development of novel peripherally targeted therapeutics for mitigating the pain of migraine.

Chapter 4

Peripheral μ-opioid receptor-mediated inhibition of calcium signaling and action potential-evoked calcium transients in primary afferent CGRP nociceptive terminals

4.1 ABSTRACT

While μ-opioid receptor (MOR) agonists remain the most powerful analgesics for the treatment of severe pain, serious adverse side effects that are secondary to their central nervous system actions pose substantial barriers to therapeutic use. Preclinical and clinical evidence suggest that peripheral MORs play an important role in opioid analgesia, particularly under inflammatory conditions. However, the mechanisms of peripheral MOR signaling in primary afferent pain fibres remain to be established. I have recently introduced a novel *ex vivo* optical imaging approach that, for the first time, allows the study of physiological functioning within individual peripheral nociceptive fibre free nerve endings in mice. In the present study, I found that MOR activation in selectively identified, primary afferent CGRP nociceptive terminals caused inhibition of N-type Ca^{2+} channel signaling and suppression of action potential-evoked Ca^{2+} transients through activation of 'big conductance' Ca^{2+}-activated K^+ channels (BK_{Ca}). In the live animal, I showed that the peripherally acting MOR agonist HS-731 produced analgesia and that BK_{Ca} channels were the major effectors of the peripheral MOR signaling. I have identified two key molecular transducers of MOR activation that mediate significant inhibition of nociceptive signaling in primary afferent terminals. Understanding the mechanisms of peripheral MOR signaling may promote the development of pathway-selective μ-opioid drugs that offer improved therapeutic profiles for achieving potent analgesia while avoiding serious adverse central side effects.

4.2 INTRODUCTION

μ-Opioid agonists remain the most effective analgesics for the treatment of both acute and chronic forms of severe pain. Unfortunately, serious centrally-mediated adverse effects such as sedation, respiratory depression, addiction, and tolerance limit their clinical use. μ-Opioid receptors (MORs) are expressed on primary afferent nociceptive fibres located in the periphery and their activation has been shown to produce clinically measurable analgesia and anti-inflammatory effects (Iwaszkiewicz et al., 2013; Stein, 2013). However, the mechanisms by which MOR signaling within the peripheral terminals of primary nociceptive neurons causes analgesia and/or anti-inflammatory effects remain to be established.

Because the small size of unmyelinated nociceptive fibres makes them inaccessible to electrophysiologcial recording with patch-clamp pipettes, I have recently introduced a novel optical imaging approach to investigate physiological functioning within peripheral nociceptive fibre free nerve endings in mice (Baillie et al., 2011; Baillie et al., 2012). My approach has opened up a new window for examining fundamental processes of pain signaling selectively within the peripheral terminals of individual unmyelinated nociceptive fibres in an intact tissue preparation. In the present study, I examine MOR activation in peripheral nociceptive terminals and describe mechanisms of Ca^{2+} signaling inhibition, suppression of action potential (AP)-evoked Ca^{2+} transients, and analgesia produced by a peripherally acting MOR agonist, HS 731 (6β-glycine substituted 14-*O*-methyloxymorphone) (Al-Khrasani et al., 2007; Furst et al., 2005; Spetea et al., 2004). My findings support the hypothesis that attacking pain at its source with opioids acting selectively in the periphery may achieve potent analgesia while avoiding adverse central side effects (Brower, 2000; Stein and Machelska, 2011; Stein et al., 2003).

4.3 MATERIALS AND METHODS

This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use. A total of 105 (both male and female) transgenic mice *Tg* [*Calca*-EGFP] (*GENSAT Project at Rockefeller University*) were used in this study. Methods have been previously described in detail (Baillie et al., 2012; Baillie et al., 2011) and are briefly outlined below.

4.3.1 Dural-skull preparation

The heads were removed from anaesthetized mice (1-4 month) and the brains carefully separated from the skulls leaving intact dura mater and arachnoid mater layers attached. Complete parietal bone dural-skull preparations were dissected and placed dural layer up in a microscope chamber and continuously perfused with oxygenated physiological saline consisting of (in mM): 125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 10 Glucose, 2 $MgCl_2$, 1.25 NaH_2PO_4, and 2 $CaCl_2$. In this preparation, individual nociceptive fibre integrity is preserved for millimeters, well protected on one side by the calvaria and by the dural layers on the other (Baillie et al., 2011).

4.3.2 Functional imaging

All functional imaging experiments were performed on unmyelinated calcitonin gene-related peptide (CGRP) nociceptive fibres identified using transgenic mice *Tg* [*Calca*-EGFP] and only a single fibre was imaged per preparation (Baillie et al., 2011). The fibres were selectively loaded with the membrane permeant high affinity Ca^{2+} indicator Rhod-2 AM (*Biotium*). Electrical stimulations were delivered within 10 μm of the CGRP nociceptive fibres via 1 μm bipolar tungsten electrodes (*WPI*). Stimulation intensity was kept just above threshold to elicit action potentials in physiological saline (140 - 180 μA for a duration of 100 μs). A single pulse

was applied once every 90 seconds for the timecourse experiments and once every 6 seconds for the continuous stimulation protocols (defined as stimulation periods > 5 minutes). During continuous fibre stimulation protocols, a series of 5 imaging acquisitions, each following a 60 second interval, were taken in order to 'snapshot' 2 action potential-evoked Ca^{2+} transient (or failure) events for quantification. Images were acquired at 20 frames/s using a high sensitivity 16-bit, 512 × 512 ImagEM EM CCD Camera (C9100-13, *Hamamatsu*) cooled to -65 °C and a minimum number of total images taken to reduce photodynamic damage. Increasing fluorescence baseline, steadily diminishing transients, and/or changes in fibre morphology were considered indicative of photodynamic damage, and fibres showing these changes were discarded. Fluorescence signals were converted to relative fluorescence changes over time and expressed in percentages, defined as $\Delta F/F = ((F_1 - B_1) - (F_0 - B_0))/(F_0 - B_0)$, where F_1 and F_0 are fluorescence in the terminal fibre at any given time point and at the beginning of the experiment, respectively, and B_1 and B_0 are the background fluorescence at any given time point and at the beginning of the experiment, respectively. Background values were taken from an adjacent area located at least 10 μm from imaged areas. To quantify the magnitude of fluorescence change, the peak amplitude of the transient was measured. During drug application experiments, responses were considered stable if < 5% variability was observed over an ~ 10 min control period without changes in baseline fluorescence. The average magnitude of the Ca^{2+} transients before drug application was set as 100%. Results are shown as means ± s.e.m. Statistical analysis was performed using an independent group *t*-test (two-tailed) for the comparison of two means and a one-way ANOVA with a Tukey's post-hoc test for three or more. Differences were considered to be significant when *$p < 0.05$ or greater; **$p < 0.01$ and # $p < 0.001$.

4.3.3 Eye-wiping trigeminal nociceptive behavioural test

The mouse eye wiping behavioural test for trigeminal nociception (Farazifard et al., 2005) was used in this study. Each mouse was given an intraperitoneal injection of (1) phosphate buffer saline (PBS) alone (n=7), (2) PBS + 18% dimethyl sulfoxide (DMSO) (n=7), (3) HS-731 (50 μg/kg) (*ChironWells GmbH*) (n=8), (4) paxilline (1 mg/kg) (*Tocris*) (n=8), (5) NS-1619 (10 mg/kg) (*Tocris*) (n=9), or (6) naloxone methiodide (20 mg/kg) (*Sigma*) (n=8). For experiments in which two drugs were administered, the first drug was injected 10 minutes prior to the second drug. Ten minutes post injection, mice were placed in a 6" × 6" × 6" (L × W × H) open-top clear container. Following a 10 minute habituation period, a 50 μl drop of 5 M NaCl was placed in one eye and the number of eye wipes with the ipsilateral paw was counted for 30 seconds from video recordings. Results are shown as means ± s.e.m. Statistical analysis was performed using an independent group *t*-test (two-tailed) for the comparison of two means and a one-way ANOVA with a Tukey's post-hoc test for three or more. The differences were considered to be significant when $*p < 0.05$ or greater.

4.3.4 Immunohistochemistry

Dural skull preparations were placed in 10% formalin for 15 minutes. Preparations were then rinsed in 0.01 M phosphate buffered saline (PBS) 3 times for 10 minutes each before being placed in a blocking solution consisting of 10% goat serum, 1% bovine serum albumin (BSA) and 0.01 M PBS containing 0.3% triton for 60 minutes. A MOR primary antibody (*Millipore AB5511*) was diluted to 1:2000 with 5% goat serum, 1% BSA, and 0.01 M PBS containing 0.3% triton and the preparations were added to this mixture and incubated at $4^{o}C$ for 24 h. Preparations were then rinsed in 0.01 M PBS containing 1% goat serum 3 times for 10 minutes each. The secondary antibody, Alexa Fluor 594 goat anti-rabbit IgG (*Invitrogen*) was diluted to

1:500 with 0.01 M PBS containing 1% goat serum and the preparations incubated at room temperature for 1 h. The preparations were then rinsed 3 times for 10 minutes each before imaging. In a series of control experiments (n=4), preparations incubated in the same solutions without the primary antibody and subsequently processed as above showed no labeling.

4.3.5 Drugs

The following drugs were added directly to the perfusate at the time points and for the durations indicated by the black bars in the figures for the timecourse experiments: HS-731 (*ChironWells GmbH*), [D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin (DAMGO) (*Tocris*), NS-1619 (*Tocris*), charybdotoxin (*Tocris*), and ω-conotoxin GVIA (*Alomone labs*). Naloxonazine dihydrochloride (*Tocris*) was added immediately after establishing a stable Ca^{2+} transient control period (naloxonazine dihydrochloride was present throughout the timecourses shown in the figures).

4.4 RESULTS

4.4.1 μ-Opioid receptor inhibition of Ca^{2+} signaling

MORs expressed on peripheral nociceptive fibres control afferent pain signaling and the local release of proinflammatory neuropeptides (Stein, 1995). CGRP is the most prevalent neuropeptide found in nociceptive primary afferents and plays an important role in pain transmission and neurogenic inflammation (Basbaum et al., 2009; McCoy et al., 2013). The trigeminal ganglion contains the highest concentration of CGRP-expressing neurons (Uddman et al., 1986), which extend long peripheral axons to densely innervate the cranial dura (Strassman et al., 2004). I used a primary antibody for the MOR and found punctate MOR immunoreactive co-localized labeling in peripheral CGRP nociceptive fibres terminating in the dura (Figure 4.1A-C). All terminal CGRP fibres examined showed MOR labeling (35 fibres from 5 animals). Functional imaging experiments showed that single AP-mediated Ca^{2+} transients were reliably evoked by a single pulse electrical stimulation delivered at distances greater than 500 μm proximal to the distal fibre terminations (Figure 4.2). Application of the selective MOR agonist DAMGO (1 μM) caused a rapid reversible inhibition in the amplitude of the AP-evoked Ca^{2+} transient (42.1 ± 3.1%; n=12) that was prevented by pre-application of the MOR antagonist naloxonazine (1 μM); Ca^{2+} transients remained at 101 ± 2.3%; n=9 under control conditions (Figure 4.3A+B). I have previously shown through a series of selective Ca^{2+} channel block experiments, that only the T- and N-type Ca^{2+} channels mediate the AP-evoked Ca^{2+} signaling in dural CGRP nociceptive fibre terminations and that G protein-coupled receptor activation caused inhibition of Ca^{2+} signaling selectively through the single N-type channel subtype (Baillie et al., 2012). Here I found that brief application of the selective N-type Ca^{2+} channel antagonist ω-conotoxin GVIA (1 μM) caused a large irreversible reduction in Ca^{2+} transient amplitude (42.6 ±

3.6%; n=6) (Figure 4.4A+B). Application of DAMGO (1 μM) in the presence of the N-type Ca^{2+} channel antagonist did not cause a further decrease (42.9 ± 4.1%; n=6) indicating that MOR inhibition of Ca^{2+} signaling in the terminals of CGRP containing nociceptive fibres occurs through inhibition of N-type Ca^{2+} channels (Figure 4.4A+B).

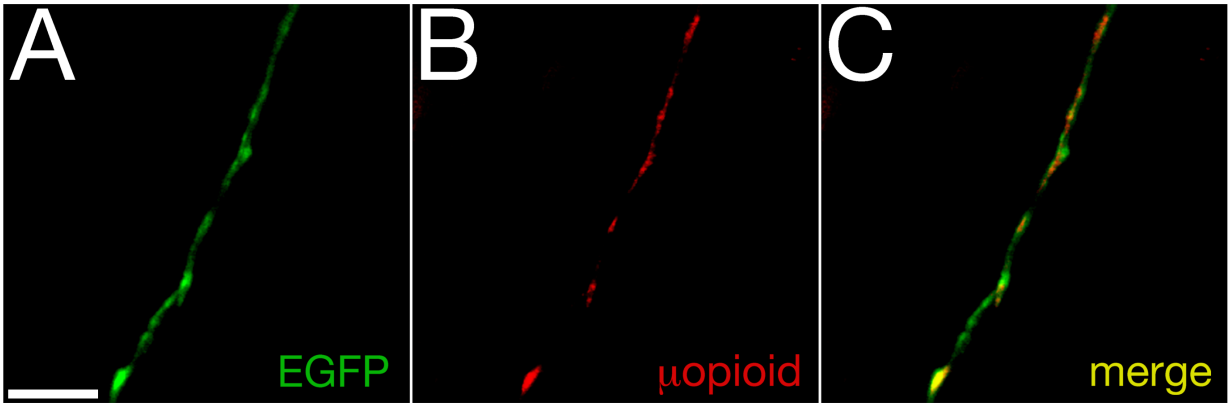

Figure 4.1. Immunohistochemical confirmation of μ-Opioid receptor expression in CGRP fibres.
A) A CGRP-EGFP terminating nociceptive fibre shows co-localized punctate labeling with a MOR antibody (B + C). Scale bar:10 μm

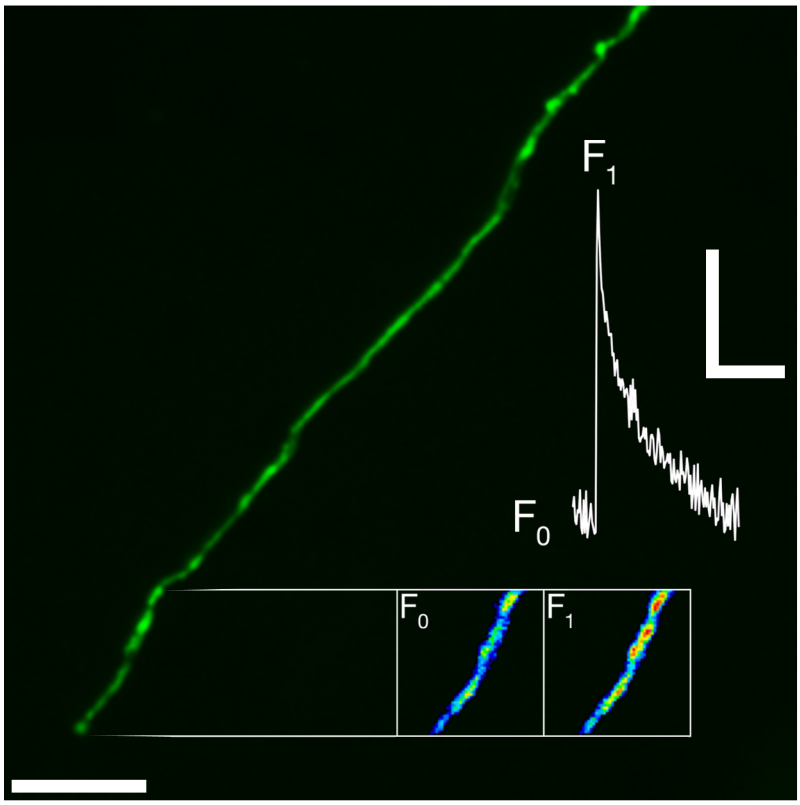

Figure 4.2. A typical peripheral CGRP fibre and corresponding action potential-evoked Ca^{2+} transient.
A CGRP-EGFP fibre showing a typical region from which fluorescent transients were acquired and quantified, along with a pseudocolour inset of baseline Rhod-2 fluorescence (F_0), and a single action potential evoked signal (F_1) with the corresponding transient above displayed. Scale bar = 20 μm, 2 s, 10% ΔF/F.

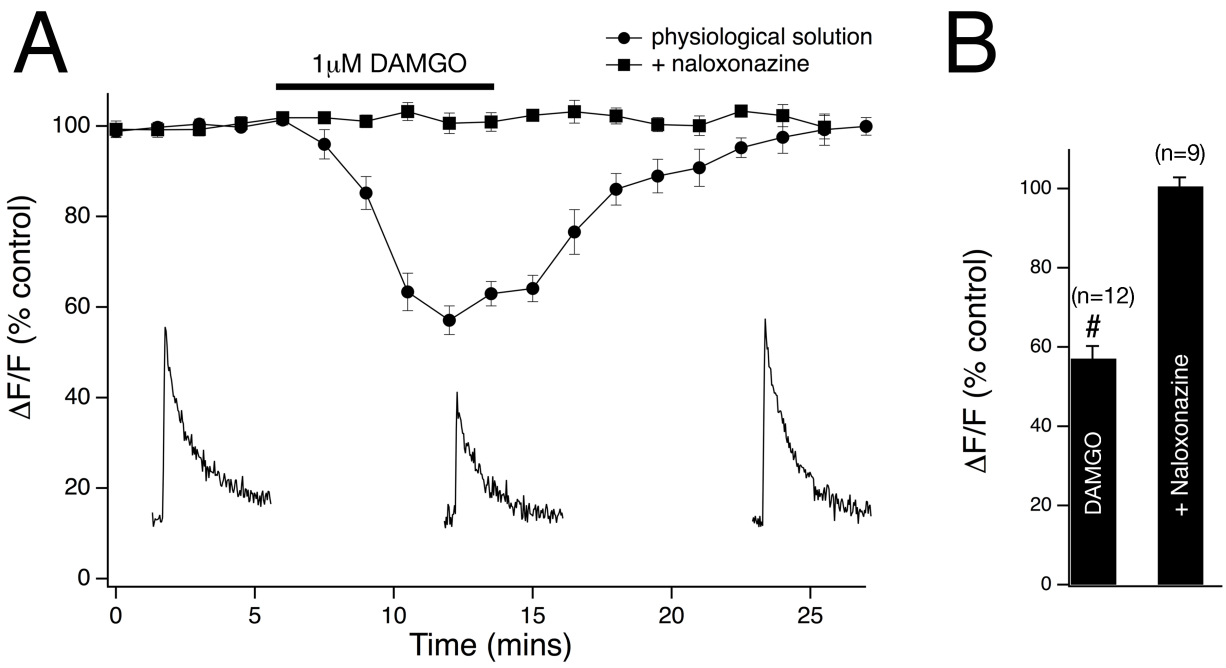

Figure 4.3. The μ-opioid receptor agonist DAMGO causes a reversible reduction in action potential-evoked Ca^{2+} transient amplitude that is blocked by the μ-opioid receptor antagonist naloxonazine.
A) Graph of Ca^{2+} transient amplitude timecourse experiments showing the effect of DAMGO. Individual example transients taken at the relative timepoints on the graph are shown below.
B) Bar graph summary of (A).

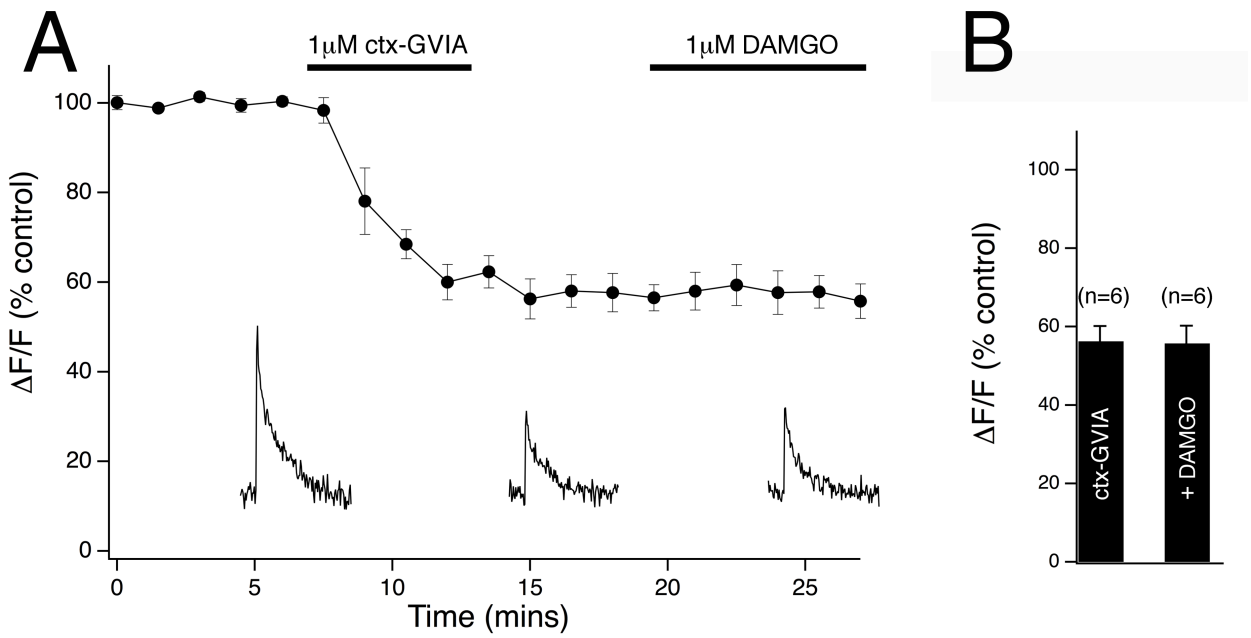

Figure 4.4. μ-Opioid receptor activation on peripheral CGRP fibres causes a decrease in action potential-evoked N-type channel mediated Ca^{2+} signaling.
A) Timecourse experiments showing the effect of the N-type Ca^{2+} channel blocker ω conotoxin-GVIA on the Ca^{2+} transient amplitudes remained unaffected with DAMGO application. Individual example transients taken at the relative timepoints on the graphs are shown below.
B) Bar graph summary.

4.4.2 μ-Opioid receptor inhibition of action potential-evoked Ca^{2+} transient firing

I next determined whether peripheral MOR activation would modulate the firing properties of the CGRP terminating nociceptive fibres. Because the small size of the unmyelinated nerve endings makes them inaccessible to conventional electrophysiological techniques, I imaged the AP-evoked Ca^{2+} transients as a means for measuring neuronal spiking (Baillie et al., 2012; Baillie et al., 2011). I consistently observed that localized DAMGO application (1 μM) to the middle region of a fibre – but not a localized puff of physiological saline to the same region – would cause AP-evoked Ca^{2+} transients to fail in an 'all-or-none' manner at locations distal to the site of drug application (Figure 4.5). I have previously shown that functional imaging could be performed over long experimental periods and that robust AP-evoked Ca^{2+} transients could be evoked without decrement, which demonstrated the stability of the preparation for examining terminal afferent physiology (Baillie et al., 2012; Baillie et al., 2011). Here I found that continuous fibre stimulation (0.167 Hz) in physiological saline elicited consistent AP-evoked Ca^{2+} transients in CGRP nociceptive fibres (AP/stimulus applied = (110/110), 100 ± 0%; n=11) (Figure 4.6A+B). However, I observed that AP-evoked Ca^{2+} transients failed frequently following application of DAMGO (1 μM). Pursuing this further, I observed that this MOR-mediated failure of AP-evoked Ca^{2+} transients persisted after the inhibition to the Ca^{2+} transient amplitude had fully recovered and this allowed us to quantify the AP-evoked Ca^{2+} transient failure rate independent of any MOR-mediated inhibition of Ca^{2+} signaling. I applied the continuous fibre stimulation protocol following DAMGO wash, (once the Ca^{2+} transients had returned to baseline control amplitudes observed prior to the DAMGO application), and found significant AP-evoked Ca^{2+} transient failures (AP/stimulus applied = (15/71), 21.1 ± 9.0%; n=6) (Figure 4.6A+B). Two fibres remained unaffected (AP/stimulus

applied = (20/20), 100 ± 0%; n=2). Pre-application of the MOR antagonist naloxonazine (1 μM) or the selective N-type Ca^{2+} channel antagonist ω-conotoxin GVIA (1 μM) prevented the MOR mediated inhibition of AP-evoked Ca^{2+} fluorescent transients (AP/stimulus applied = (76/76), 100 ± 0%; n=6 and (66/66), 100 ± 0%; n=6 respectively) (Figure 4.6A+B).

Ca^{2+}-activated K^+ channels (K_{Ca}) activate in response to both membrane depolarization and an elevation of cytosolic Ca^{2+} (Fakler and Adelman, 2008). They play key roles in the control of AP duration, firing frequency, and the generation of spike-frequency adaptation. Their modulation by a range of transmitters is a key determinant in the regulation of neuronal membrane excitability (Sah, 1996). Based on biophysical as well as pharmacological properties K_{Ca} are separated into two families: small (SK_{Ca}) and big (BK_{Ca}) conductance channels (Berkefeld et al., 2010). I found that the specific inhibitor of BK_{Ca} channels charybdotoxin (50 nM) caused an increase in the amplitude of the AP-evoked Ca^{2+} transient (126 ± 8.6%; n=6) (Figure 4.7A). Application of DAMGO (1 μM) in the presence of Charybdotoxin caused a rapid inhibition in the amplitude of the AP evoked Ca^{2+} transient by 41.6 ± 7.4% (n=6) that was fully reversible with washing (128 ± 14.5%, n=6) (Figure 4.7A+B). Two fibres did not show an initial increase in the amplitude of the AP-evoked Ca^{2+} transient in charybdotoxin and therefore DAMGO application was not pursued. The selective inhibitor of SK_{Ca} apamin showed no effect (data not shown). I then applied continuous fibre stimulation in the presence of charybdotoxin and following DAMGO wash out and found consistent AP-evoked Ca^{2+} fluorescent transients (AP/stimulus applied = (87/88), 98.8 ± 1.9%; n=6) (Figure 4.8A+B). Apamin showed no effect (data not shown). The selective BK_{Ca} channel activator NS-1619 (10 μM) caused frequent failure of the continuous fibre stimulation-evoked Ca^{2+} transients (AP/stimulus applied = (19/90), 21.1 ± 5.3%; n=6) (Figure 4.8A+B).

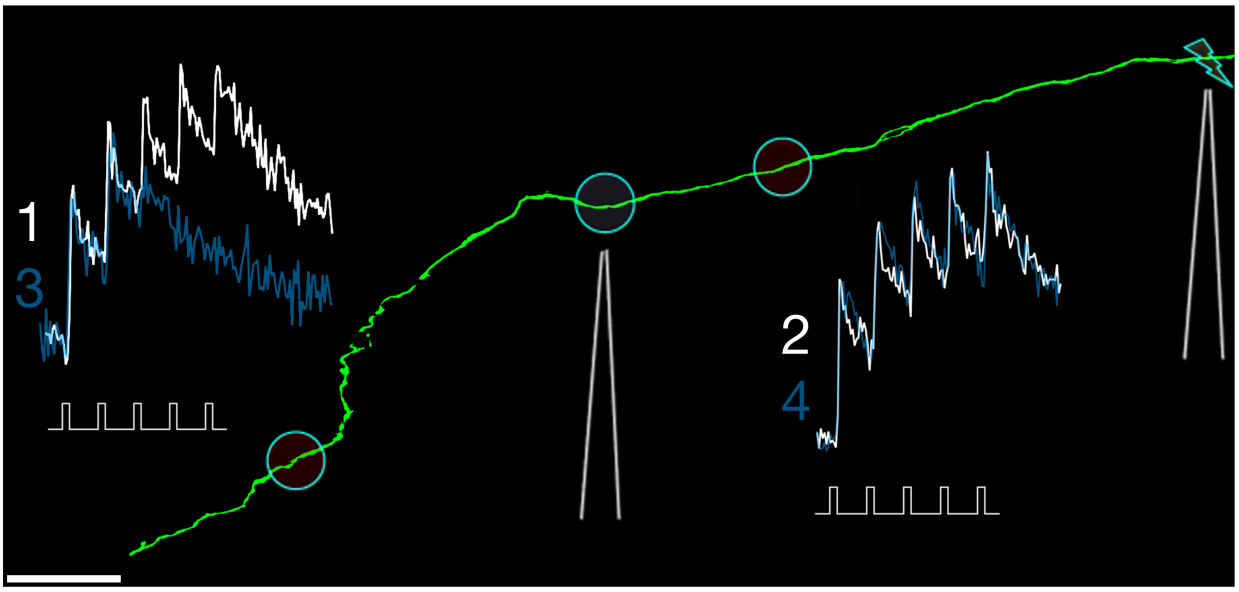

Figure 4.5. Localized DAMGO application causes action potential failure in an 'all-or-none' manner.
Image illustrating AP propagation failures observed after localized DAMGO application. In control conditions, a train of five 1 Hz stimulations caused AP evoked facilitating Ca^{2+} transients at both distal (1) and proximal (2) imaging locations. After a brief localized puff of DAMGO (centre circle), Ca^{2+} transients at the distal (3) imaging location fail in an 'all-or-none' manner but do not at the proximal (4) location ('all-or-none' AP failures seen at proximal locations when the fibre was stimulated at the distal location and were observed with a range of stimulation paradigms). Scale bar = 50 μm.

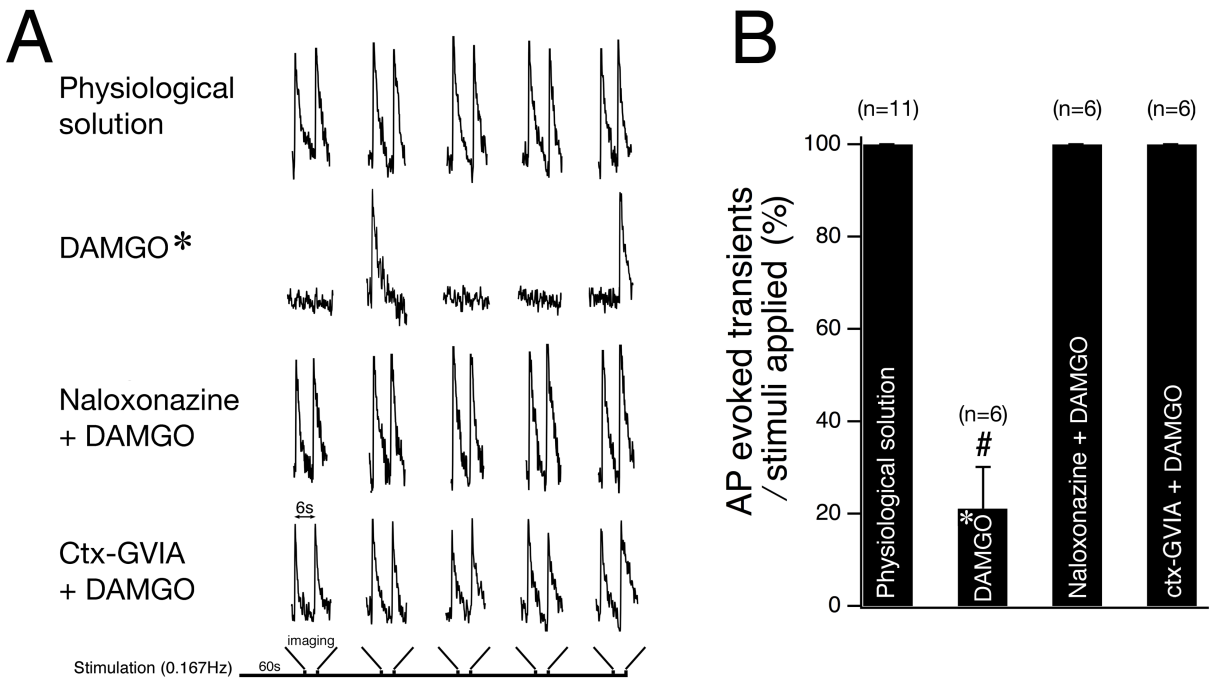

Figure 4.6. The μ-opioid receptor agonist DAMGO causes action potential failure during continuous fibre stimulation that is prevented by preapplication of the μ-opioid receptor antagonist naloxonazine or the N-type Ca^{2+} channel blocker conotoxin-GVIA.
A) In control conditions, continuous fibre stimulation elicits consistent AP firing observed as non-failing Ca^{2+} transients. APs fail often during continuous fibre stimulation experiments when DAMGO had been previously applied to a fibre and after which the Ca^{2+} transients had returned to baseline control amplitudes (asterisk used to distinguish this condition from in the presence of DAMGO). Non-failing Ca^{2+} transients were observed when naloxonazine or ω conotoxin-GVIA was applied before DAMGO. **B**) Bar graph summary quantifying the number of AP-evoked Ca^{2+} transients as a percentage of stimuli applied during the conditions shown in (A).

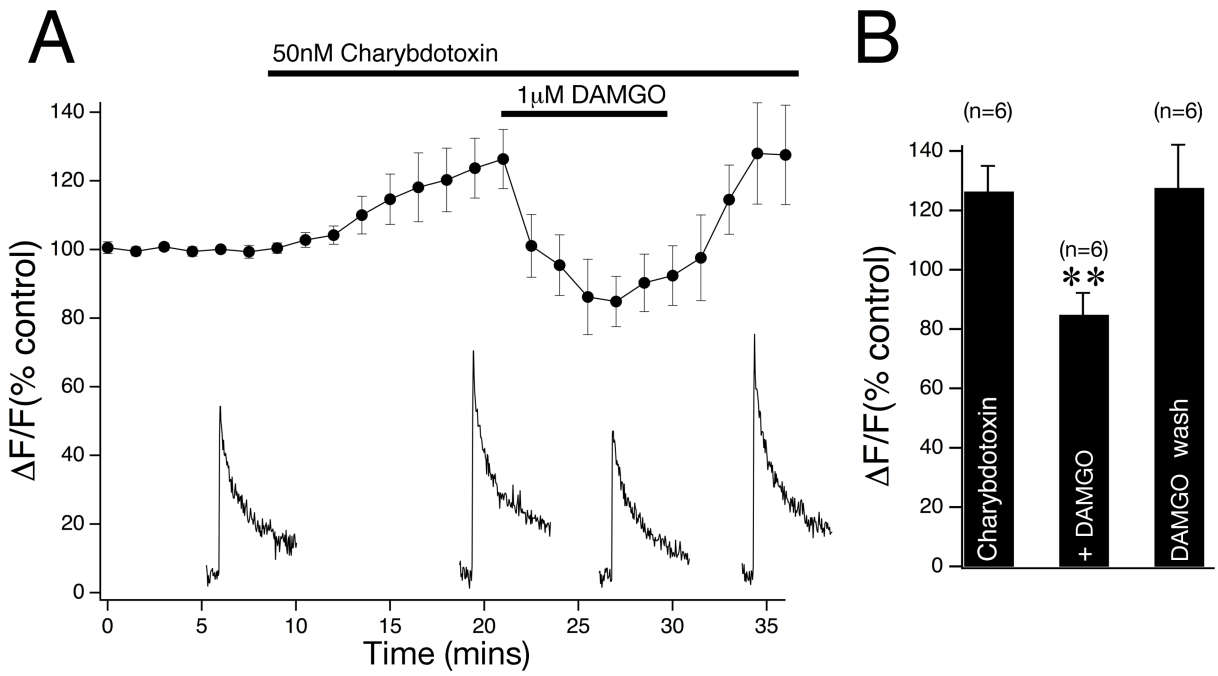

Figure 4.7. The BK_{Ca} channel antagonist charybdotoxin causes an increase in action potential-evoked Ca^{2+} transient amplitude that is reduced by DAMGO.
A) Graph of Ca^{2+} transient amplitude timecourse experiments showing the effect of DAMGO application in the presence of the specific inhibitor of BK_{Ca} channels charybdotoxin. Individual example transients taken at the relative timepoints on the graph are shown below. **B**) Bar graph summary of (A).

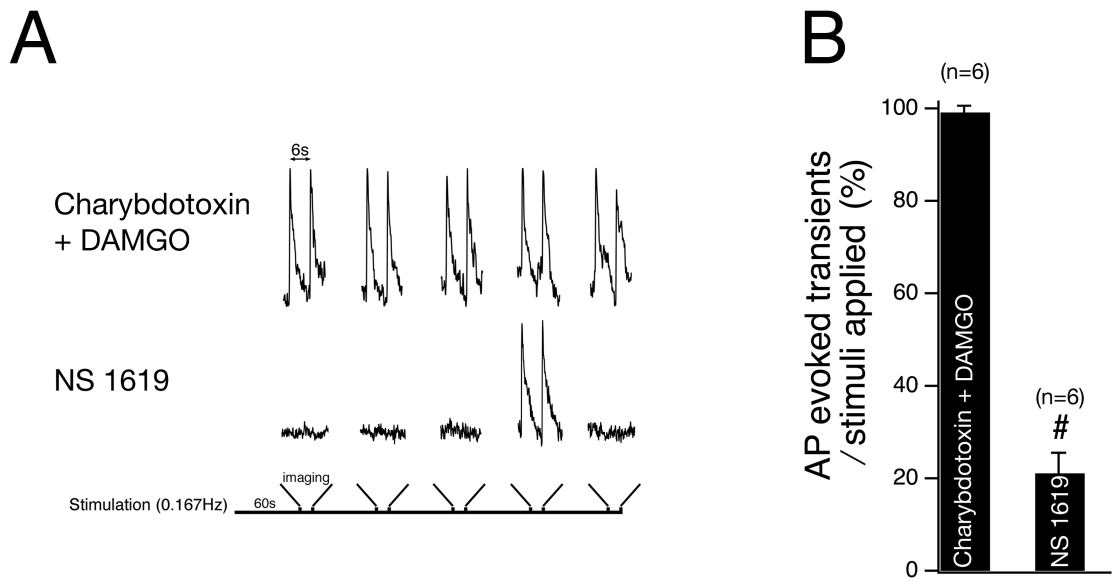

Figure 4.8. The BK_{Ca} channel antagonist charybdotoxin prevents DAMGO-mediated action potential failure and the BK_{Ca} channel agonist NS 1619 mimics the action potential failure observed with DAMGO application.

A) Sustained AP firing was observed when charybdotoxin was applied before DAMGO, but APs fail often in experiments with the BK_{Ca} channel activator NS-1619 applied alone. **B**) Bar graph summary of (A).

4.4.3 Peripheral μ-opioid receptor mediated antinociception

I examined whether MOR activation by a peripherally acting MOR agonist would cause inhibition of Ca^{2+} signaling and AP-evoked Ca^{2+} transients in the CGRP terminating nociceptive fibres. The 6 β-glycine substituted derivative of 14-*O*-methyloxymorphone (HS-731) has been described as a potent MOR agonist that does not readily cross the blood brain barrier, and induces peripherally-mediated antinociceptive actions in various pain models (Al-Khrasani et al., 2007; Bileviciute-Ljungar et al., 2006; Furst et al., 2005). Application of HS-731 (1 μM) caused a rapid reversible inhibition in the amplitude of the AP evoked Ca^{2+} transient (40.0 ± 3.6%; n=5) that was prevented by pre-application of the MOR antagonist naloxonazine (1 μM) (103 ± 2.1%; n=5) (Figure 4.9+4.10A). I then applied the continuous fibre stimulation protocol following a HS-731 wash out period, after which the Ca^{2+} transients had returned to the baseline control amplitudes before HS-731 application, and found that AP-evoked Ca^{2+} transients failed frequently (AP/stimulus applied = (17/85), 20.0 ± 6.7%; n=7) (Figure 4.10B). Pre-application of the MOR antagonist naloxonazine (1 μM) prevented the MOR mediated inhibition of AP-evoked Ca^{2+} transients (AP/stimulus applied = (63/63), 100 ± 0%; n=5) (Figure 4.10B).

To determine whether HS-731 produced antinociception I used the mouse eye wipe in response to NaCl application behavioural test for trigeminal nociception (Farazifard et al., 2005). Animals given control vehicle intraperitoneal injections exhibited 16.2 ± 1.0 (n=14) eye wipes with the ipsilateral paw in 30 seconds. Intraperitoneal injection of HS-731 (50 μg/kg) caused a significant decrease in the number of mouse eye-wipes (9.9 ± 2.1; n=8) that was prevented by a prior injection of the peripherally acting MOR antagonist naloxone methiodide (20 mg/kg) (16.0 ± 1.9; n=8) (Figure 4.11). Intraperitoneal injection of the potent blocker of BK_{Ca} channels

paxilline (1 mg/kg) did not change the number of eye wipes (17.7 ± 1.0; n=8) compared to control conditions, but prevented the HS-731 induced reduction (15.6 ± 1.9; n=8). Injection of the BK_{Ca} channel activator NS-1619 (10 mg/kg) alone mimicked the decrease in the number of eye wiping seen with HS-731 (12.0 ± 1.9; n=9) (Figure 4.11).

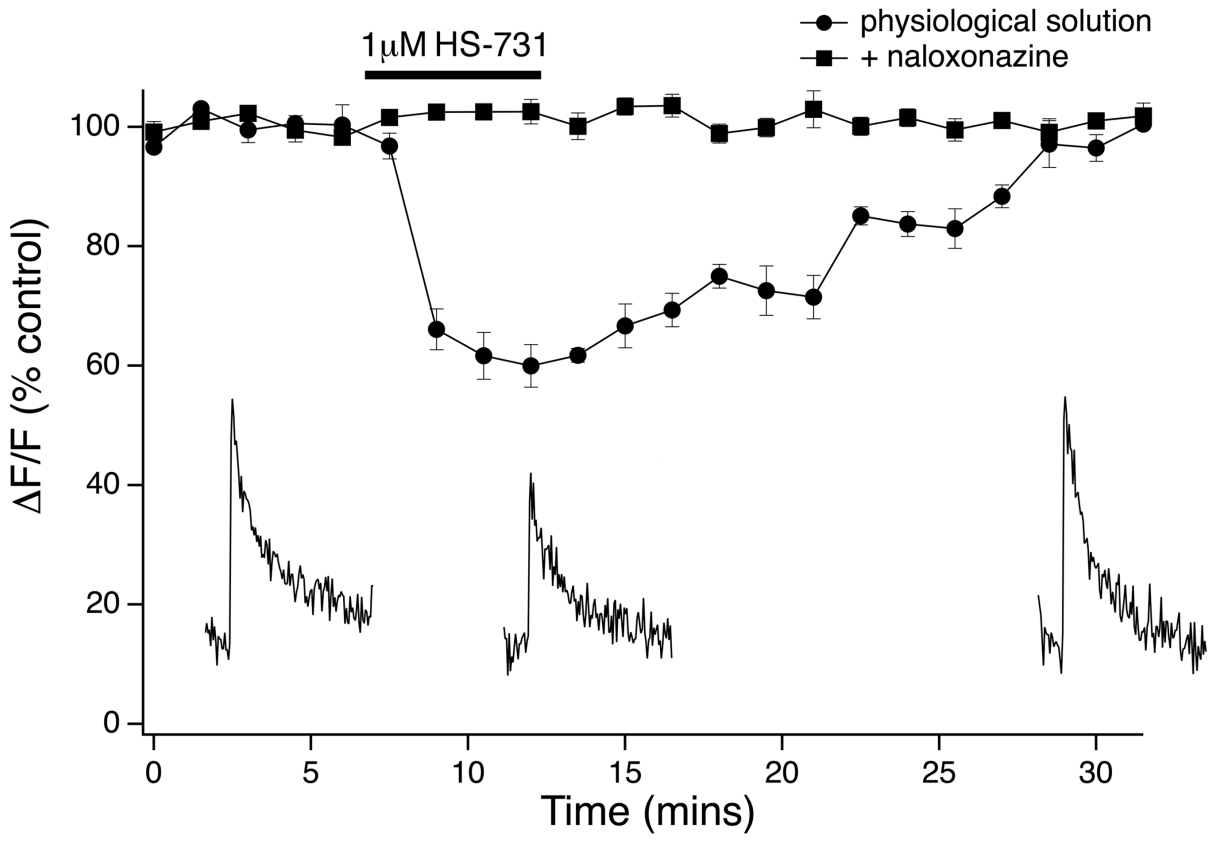

Figure 4.9. The peripherally restricted MOR agonist HS-731 produces a reduction in action potential-evoked Ca^{2+} transient amplitude similar to DAMGO.
Graph of Ca^{2+} transient amplitude timecourse experiments showing the effect of HS-731. Individual example transients taken at the relative timepoints on the graph are shown below.

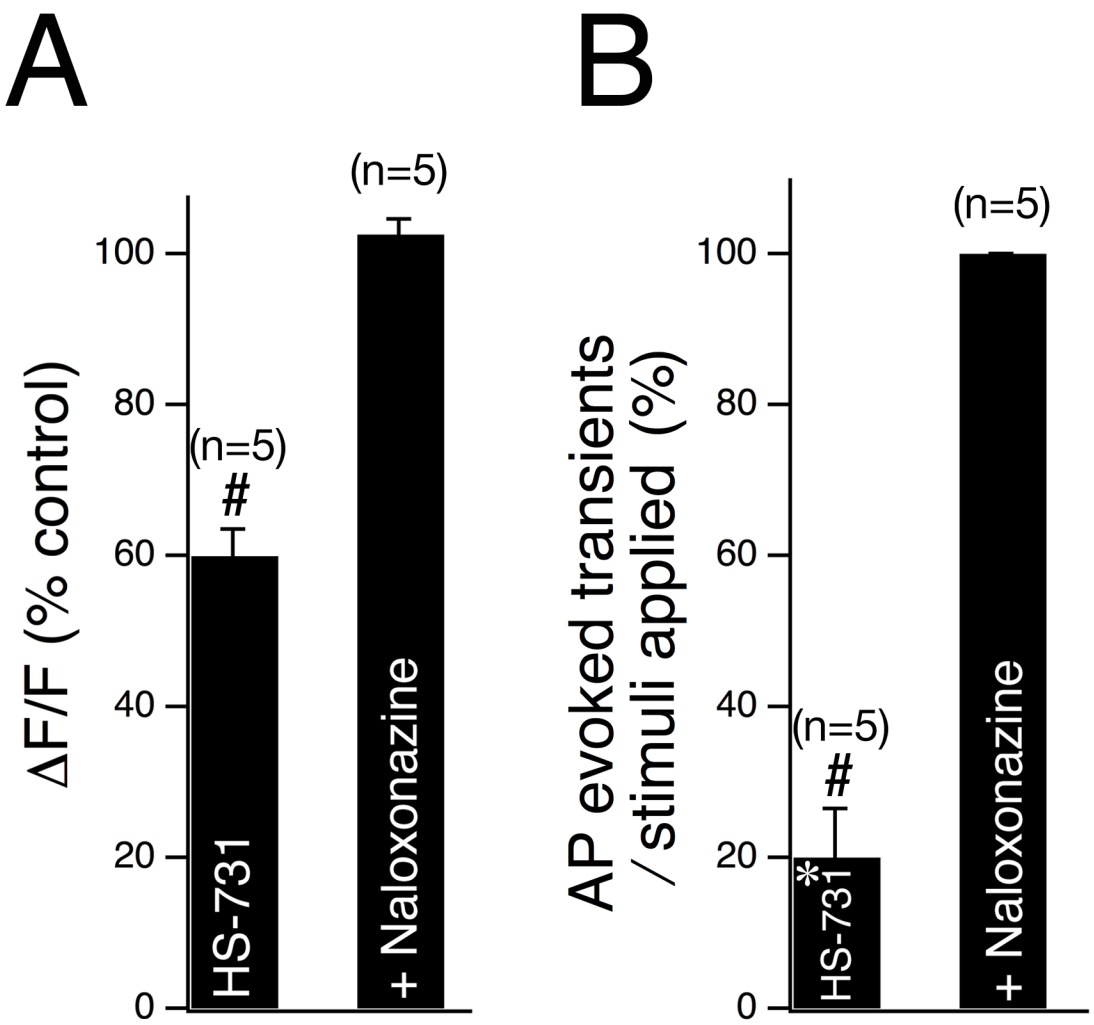

Figure 4.10. The peripherally restricted MOR agonist HS-731 causes a reduction in action potential-evoked Ca^{2+} transient amplitude and action potential failure similar to DAMGO. **A**) Bar graph summary showing that the HS-731 reduction in the amplitude of the Ca^{2+} transient was completely blocked by naloxonazine. **B**) Bar graph summary quantifying the number of AP evoked Ca^{2+} transients during continuous fibre stimulation as a percentage of stimuli applied for experiments with previous application of HS-731 (asterisk used to distinguish this condition from in the presence of HS-731) and MOR block with naloxonazine.

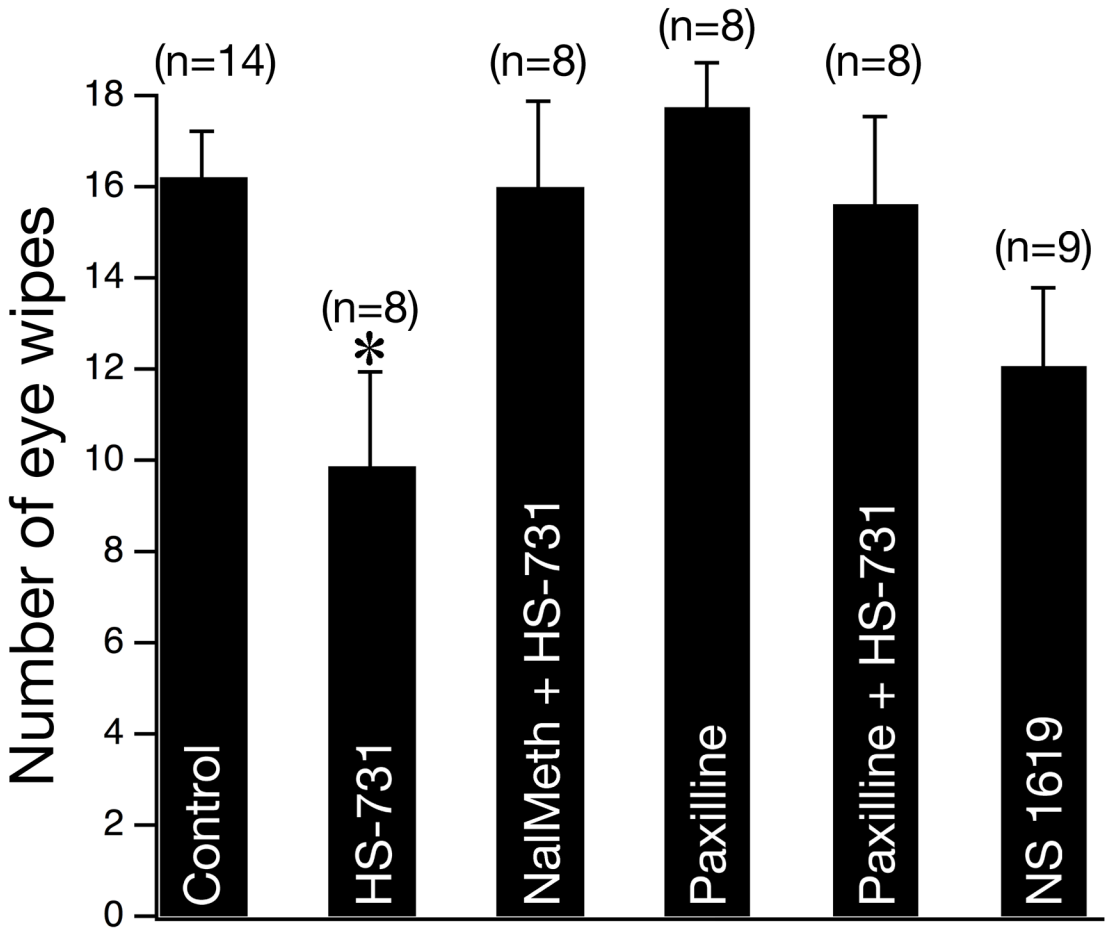

Figure 4.11 The peripherally restricted MOR agonist HS-731 caused antinociception in the mouse eye-wiping behavioural test of trigeminal nociception.
Bar graph summary quantifying the eye-wiping behavioural experiments. The peripherally acting MOR agonist HS-731 caused a significant decrease in the number of eye-wiping that was normalized from a preceding injection of the peripheral MOR antagonist naloxone methiodide (NalMeth). The potent blocker of BK_{Ca} channels paxilline prevented the HS-731 induced reduction, while injection of the BK_{Ca} channel activator NS-1619 alone mimicked the decrease in the number of eye-wipes seen with HS-731. *$p < 0.05$.

4.5 DISCUSSION

In the present study I discovered that peripheral MOR activation in individual nociceptive CGRP terminals has a dual modulatory effect, that is, inhibition of N-type Ca^{2+} channel signaling and BK_{Ca} channel-mediated suppression of AP-evoked Ca^{2+} transients. I extended these observations to the whole animal and showed that the peripherally acting MOR agonist HS-731 produced analgesia that was prevented by a potent antagonist of BK_{Ca} channels and mimicked by an activator of BK_{Ca} channels administered alone.

The terminals of CGRP-containing primary afferent nociceptors respond to noxious stimuli not only by transmitting APs toward the central nervous system, but by releasing CGRP locally in the periphery to promote inflammation (Gold and Gebhart, 2010; Jancsó, 2009; Richardson and Vasko, 2002). CGRP is among the most potent vasodilators (Brain et al., 1985) and its peripheral release plays a major role in inflammatory pain by mediating neurogenic vasodilation (Meyer et al., 2005). N-type Ca^{2+} channels control neurotransmitter release from peripheral sensory neurons (Snutch, 2005). Given the significant contribution of the N-type Ca^{2+} channels to the Ca^{2+} signal in dural nociceptive CGRP fibres (~40%), their selective inhibition through MOR activation should dramatically inhibit CGRP release, since neurotransmitter release shows a non-linear dependence on Ca^{2+} influx through voltage dependent Ca^{2+} (Zucker, 1993). My present findings suggest that MORs located on CGRP fibres in the periphery may be an attractive target for therapeutic intervention to reduce neurogenic vasodilation and inflammatory pain states.

BK_{Ca} channels are expressed in peripheral CGRP nociceptive neurons (Lu et al., 2014) and are thought to be important regulators of pain signaling in afferent nociceptive fibres (Tsantoulas and McMahon, 2014; Waxman and Zamponi, 2014). A substantial reduction in both

the mRNA and protein levels of BK_{Ca} channels in primary afferent neurons has been seen in a model of neuropathic pain (Chen et al., 2009). BK_{Ca} channel block has been shown to prolong the duration of APs and increase firing frequency, while their activation suppressed AP firing (Zhang et al., 2003). In this study I found that the profound MOR mediated suppression of AP-evoked Ca^{2+} transients (~80%) was completely blocked with BK_{Ca} channel antagonism and mimicked by BK_{Ca} channel activation alone. Thus I have identified BK_{Ca} channels as mediators of peripheral MOR activation that causes suppression of AP firing in CGRP nociceptive fibres. My findings suggest that peripheral MORs located on CGRP fibres acting through BK_{Ca} channels, may represent a new therapeutic target to effectively quell the increased excitability and ectopic AP firing seen in primary afferent nociceptive fibres in neuropathic pain states (Cohen and Mao, 2014). Considering the efferent functioning of the nociceptive fibres, the dramatic suppression of AP firing from peripheral MOR activation would be expected to considerably reduce the release of CGRP. Indeed, it has recently been shown that NS1619 dose dependently inhibited neurogenic dural vasodilation in a model of trigeminovascular nociception (Akerman et al., 2010).

I found that the peripherally acting MOR agonist HS-731 caused inhibition of Ca^{2+} signaling (~40%) and suppression of AP-evoked Ca^{2+} fluorescent transients (~80%) in CGRP fibres to the same extent as the prototypical MOR agonist DAMGO. To evaluate the analgesic potential of HS-731 I used the mouse eye wiping behavioural test for trigeminal nociception. I chose this model for the current study because the cornea is extremely densely innervated by a network of CGRP nociceptive fibres that are derived from the same ophthalmic branch of the trigeminal nerve that innervates the dura (Muller et al., 2003). HS-731 produced significant antinociception by suppressing the pain evoked eye wiping behaviour by ~40%. Support for my

proposal that BK_{Ca} channels are peripheral MOR effectors, analgesia was prevented with BK_{Ca} channel antagonism and mimicked by BK_{Ca} channel activation. These findings suggest that potent analgesia may be achieved with peripherally selective therapeutics targeting MORs located outside the central nervous system, thus eliminating the serious side effects that accompany conventional opioid interventions.

Chapter 5

General discussion

The work in this dissertation outlined the development of a novel preparation for the study of nociceptive signaling within the peripheral terminals of individual nociceptors and highlighted the utility of this preparation by determining the effects two analgesics had on signaling in the peripheral terminals of CGRP containing nociceptive fibres.

5.1 Major thesis findings

1. The development of the dural-skull preparation allows, for the first time, the study of nociceptive signaling within the peripheral terminals of nociceptors while they reside in the parietal dura mater, undisturbed, protected by the meningeal dura mater on one side and the calvaria on the other.

2. The commonly used anti-migraine drug, sumatriptan, which via activation of 5-HT_{1D} receptors, inhibited N-type Ca^{2+} channels resulting in a reduction in the amount of Ca^{2+} entering the nociceptive terminal during an action potential.

3. In the peripheral terminals of CGRP-containing nociceptive fibres μ-opioid receptor activation resulted in a reduction in Ca^{2+} signaling through inhibition of N-type Ca^{2+} channels similar to the sumatriptan study as well as caused a frequency-dependent suppression of action potential firing that occurred through activation of the BK Ca^{2+}-activated K^{+} channel.

5.2 Functional imaging within individual pain fibres *ex vivo* with optical microscopy.

I have introduced an experimental imaging approach to directly study physiological functioning within selectively identified nociceptive fibre free nerve endings that terminate in the cranial dura mater. While the peripheral nociceptors innvervating the dura mater are far too small to study using conventional electrophysiological technqiues such as intracellular recording with patch clamp pipettes, the relatively thin, translucent cellular constituents of the intracranial meninges makes it ideal for experiments involving high-resolution optical imaging (Figure 5.1). This *en bloc* dural skull preparation can be performed in rats and mice, preserves the integrity of nociceptive processes for millimeters and offers ideal tissue conditions for optical microfluorometric imaging.

The dural skull preparation introduced here allows for the identification of myelinated Aδ-fibre and unmyelinated C-fibre nociceptors. The optical properties of the dura mater allow for identification of myelin with simple transmitted light, which I confirmed was truly myelin with the selective myelin stain Fluoromyelin Red® (Invitrogen). The dural skull preparation also allows for the study of both main subpopulations of C-fibres; peptidergic with the use of a transgenic CGRP-EGFP mouse (Transgenic Tg [Calca-EGFP] (GENSAT Project at Rockefeller University)) and non-peptidergic with the application of a fluorescent conjugated isolectin B4 that selectively bind to membrane-associated glycoconjugates that exist only in non-peptidergic nociceptors (Soyguder, 1999).

Using a brief application of the high affinity Ca^{2+} indicator Rhod2-AM and electrical stimulation I was able to show that single action potential-induced Ca^{2+} transients could be elicited with high signal-to-noise ratios in individual C-fibres with no decrement in size for

extended periods of time. To my knowledge this is the first study to show high-resolution functional imaging selectively within individual nociceptive fibre terminations.

The cellular make up of the dural-skull preparation also makes it ideal for experiments using molecular uncaging. I was able to couple a 355 nm wavelength laser to a wide field epifluorescence microscope to allow focal uncaging in and near individual nociceptive terminals. The use of molecular uncaging allows localized and precise control of molecule application (Amatrudo et al., 2014; Pettit et al., 1997).

Finally I was able to provide direct evidence of the axon reflex in a single CGRP peptidergic nociceptor. The axon reflex, first proposed by (Bruce, 1913) suggests that both afferent and efferent activity can occur in the same primary sensory neuron at a point of bifurcation. Activation of one branch of a nociceptor results in action potentials that travel afferently back to the CNS but also efferently down other branches at points of bifurcation. The efferent signaling that occurs at a point of bifurcation results in the release of neuropeptides such as CGRP and substance P (Richardson and Vasko, 2002), which leads to neurogenic inflammation and peripheral sensitization (Foreman, 1987).

Although the axon reflex was first proposed over 100 years ago, this is the first study to my knowledge that provides direct evidence of its existence in a single branching nociceptor as opposed to bundles of axons where phenomena such as ephaptic transmission could account for previous observations.

Signaling in the afferent terminals of nociceptors depends on the distribution, density and biophysical properties of molecular transducers and ion channels that exist within them (Woolf and Ma, 2007), which likely differ from the cell bodies of nociceptors in which much of our current understanding of nociception has been obtained (Kostyuk and Kostyuk, 2009).

High-resolution functional optical imaging in the en bloc dural-skull preparation now provides an opportunity to begin to study the fundamental physiological processes of activation, transduction, sensitization, and propagation of the nociceptive signal in peripheral pain fibre terminations. This opens up a new window for examining physiological functioning in peripheral nociceptive fibres and to advance our understanding of the peripheral processes involved in pain pathophysiology.

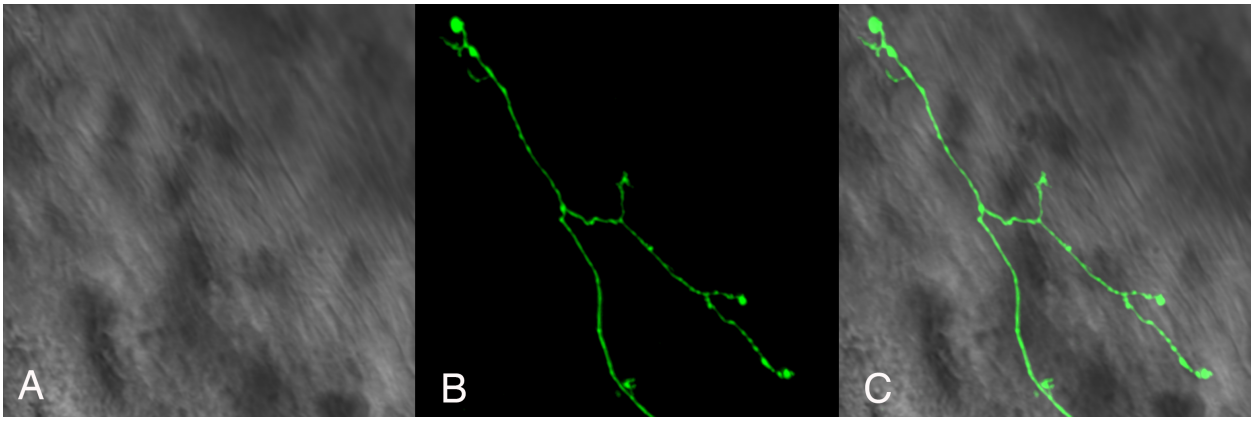

Figure 5.1. High-resolution image of terminating CGRP nociceptive fibre in the cerebral meninges.
A) Two-photon IR-transmitted image of the relatively translucent meninges in the dural-skull preparation. **B**) Two-photon image of a terminating CGRP nociceptive fibre from CGRP-EGFP transgenic mouse. **C**) Merge of (A+B).

5.3 Sumatriptan inhibition of N-type calcium channel mediated signaling in dural CGRP terminal fibres.

Since the anatomical locus of the triptan's anti-migraine effects have been long debated (Hoskin and Goadsby, 1998; Humphrey and Goadsby, 1994) I wished to test its effect on nociceptive signaling within the CGRP nociceptive terminals, which are believed to play a large role in the pathophysiological mechanisms of migraine headache (Durham, 2006).

I found a concentration-dependent decrease in the amplitude of action potential-evoked Ca^{2+} transients during bath application of the 5-$HT_{1B/D}$ agonist sumatriptan. Through the use of separate 5-HT_{1B} and 5-HT_{1D} antagonists I determined that sumatriptan's effect on Ca^{2+} transient amplitude was mediated through activation of the 5-HT_{1D} receptors specifically.

Next I performed a series of Ca^{2+} channel blocking experiments to determine which Ca^{2+} channel(s) are being inhibited by the activation of the 5-HT_{1D} receptor and found interesting results, the first being the types of voltage gated Ca^{2+} channels present in the peripheral terminals of CGRP nociceptive fibres. Studies of cultured nociceptors have found the presence of a multitude of voltage gated Ca^{2+} channels including, P/Q, L, N, R and T (Yusaf et al., 2001) and a study of sumatripan's effect on CGRP release from cultured TRG neurons found that P/Q, L and N-type Ca^{2+} channels are all inhibited during 5-$HT_{1B/D}$ activation resulting in a decrease in CGRP release (Amrutkar et al., 2011). Through Ca^{2+} channel blocking experiments in the peripheral terminals of CGRP nociceptive fibres I found that only T-type and N-type Ca^{2+} channels are activated during an electrically-evoked action potential and that sumatriptan's reduction in Ca^{2+} transient amplitude was due solely to inhibition of the N-type Ca^{2+} channel.

To my knowledge this is the first study to show that triptans inhibit Ca^{2+} signaling selectively through a single Ca^{2+} channel subtype, the N-type Ca^{2+} channel. Although I did not

have a measurement for the release of neuropeptides from the CGRP nociceptive fibres that I was studying, it can be inferred that the (~40%) reduction in Ca^{2+} signal through the activation of 5-HT_{1D} receptors should dramatically inhibit neurotransmitter release, since release shows a non-linear dependence on Ca^{2+} influx through voltage dependent Ca^{2+} channels (Bollmann and Sakmann, 2005; Dodge and Rahamimoff, 1967; Schneggenburger and Neher, 2005). N-type Ca^{2+} channels have also been proposed to control neurotransmitter release from peripheral sensory neurons (Snutch, 2005) lending more evidence that neuropeptide release would be reduced through N-type Ca^{2+} channel inhibition.

When a migraine is initiated and peripheral CGRP nociceptive fibres become activated it is possible that through the efferent signaling component of the axon reflex that CGRP release from activated terminals results in a state of neurogenic inflammation, peripheral sensitization and allodynia causing the meningeal nociceptors to then react to normally non-painful vascular stimuli and result in the long-lasting painful phase of migraine (Olesen et al., 2009). Thus triptans may relieve the painful state of migraine by inhibiting N-type Ca^{2+} channels leading to a reduction in CGRP or substance P release, resolving neurogenic inflammation, peripheral sensitization and the corresponding allodynia allowing nociceptive thresholds to return to baseline and the cessation of their activation.

Aside from acting on the peripheral terminals of nociceptors it has also been proposed that triptans may cause migraine relief though arterial vasoconstriction (Ahn and Basbaum, 2005). It is worth noting, in experiments not included in the chapter publication, I tested the effect bath applied sumatriptan had on dural arteriole diameter in the dural-skull preparation and found no vascular changes with a concentration of sumatriptan that caused the maximal change in transient amplitude (~20 μM). To confirm vasculature was still viable in the dural skull

preparation, 1 μM norepinephrine was applied in a similar manner and resulted in repeatable vasoconstriction of dural arterioles. These results favour the idea that the peripheral terminals of nociceptors are an anatomical locus of triptans in relieving migraine pain.

5.4 Peripheral μ-opioid receptor mediated inhibition of calcium signaling and action potential- evoked calcium transients in primary afferent CGRP nociceptive terminals.

In this study I wished to determine the effect μ-opioid receptor activation had on nociceptive signaling in the peripheral terminals of CGRP fibres. First I found nearly identical results to the sumatriptan study in that activation of μ-opioid receptors by a selective μ-agonist DAMGO or novel peripherally-restricted, selective μ-opioid agonist, HS-731 (Furst et al., 2005) caused an approximately 40% reduction in the amplitude of AP-evoked Ca^{2+} transients and that this reduction was mediated through the inhibition of N-type Ca^{2+} channels only. The reduction in Ca^{2+} entry through μ-opioid receptor activation would likely play a role in the reduction of inflammatory pain. During nociceptor activation signals travel not only afferently to the CNS but also efferently at points of bifurcation leading to release of neuropeptides at the peripheral terminals. It can be inferred that the large reduction in Ca^{2+} entry through μ-opioid receptor activation would result in a reduction in CGRP release from the peripheral terminals thus relieving the neurogenic inflammation and reducing inflammatory pain that occurs as a result of CGRP release (Richardson and Vasko, 2002).

A novel finding in this study was the observation of frequency-dependent AP-evoked Ca^{2+} transient failure after the application of μ-opioid receptor agonists. Following μ-opioid receptor activation I observed that AP-evoked Ca^{2+} transients fail in an 'all-or-none' manner when an increase in frequency of stimulation was applied. This failure in AP firing was attributed to activation of the large conductance Ca^{2+}-activated K^+ channel (BK_{Ca}). BK_{Ca} channels have been shown to be expressed on CGRP-containing primary afferents (Lu et al., 2014) and are believed to be important in pain signaling within afferent nociceptive fibres (Tsantoulas and McMahon, 2014; Waxman and Zamponi, 2014).

BK_{Ca} channel block has been shown to prolong the duration of APs and increase firing frequency, while their activation suppressed AP firing (Zhang et al., 2003). These findings were both observed in this study with an increase in amplitude and width of AP-evoked Ca^{2+} transients observed after BK_{Ca} antagonism by charybdotoxin indicating an increase in AP duration and with a suppression in AP-evoked Ca^{2+} transients in an all-or-none manner via activation of the μ-opioid receptor or by direct activation of the BK_{Ca} channel by the agonist NS-1619, indicating AP failure. Antagonism of the BK_{Ca} channel by charybdotoxin also prevented the μ-opioid receptor mediated frequency-induced failure of AP-evoked Ca^{2+} transients lending more evidence to BK_{Ca} being responsible for the failure of AP firing.

Interestingly I was able to extend results observed *ex vivo* in the dural skull preparation, to *in vivo* using the mouse eye wiping behavioural test for trigeminal nociception. μ-opioid receptor activation resulted in a ~40% reduction in pain-evoked eye wiping. This analgesia through μ-opioid receptor activation was prevented by blocking BK_{Ca} channels and mimicked by direct activation of BK_{Ca} channels lending evidence to the proposal that BK_{Ca} are modulated by μ-opioid receptor activation.

To my knowledge this is the first study to identify BK_{Ca} channels as mediators of peripheral μ-opioid receptor activation and that μ-opioid receptor mediated BK_{Ca} activation leads to suppression of AP firing in CGRP nociceptive fibres. These findings suggest a new therapeutic pathway through which μ-opioid receptor activation acts in relieving pain and a potential therapeutic target in the development of more specific, peripherally acting analgesics that avoid the unwanted central side effects that are commonly associated with current analgesics.

5.5 Limitations

Like all current methods for studying cellular nociception, the *ex vivo* dural skull preparation has some limitations, one being the use of chemical Ca^{2+} indicators. While day-to-day loading of dural CGRP nociceptors with the cell permeable high affinity Ca^{2+} indicator Rhod-2 AM, consistently resulted in a healthy preparation in which single afferents could be stimulated for hours. Occasionally, particularly when a new lot of Ca^{2+} indicator was purchased, loading times had to be slightly adjusted to prevent both excess loading of background cells (resident macrophages and dendritic cells) reducing signal-to-noise and over-loading of the nociceptors themselves leading to cell death.

This limitation, while minor, will hopefully be extinguished with the use of the newly developed genetically encoded Ca^{2+} indicator GCAMP6 (Chen et al., 2013). This newly developed genetically encoded indicator has a 10-fold improvement in Ca^{2+} sensitivity over the previous version, GCAMP3 and surprisingly was shown to exceed one of the commonly used chemical indicators, Oregon Green Bapta-1 AM in dissociated rat hippocampal neurons (Figure 5.2).

The cross of two transgenic mice, one that expresses cre-recombinase in TRPV1 containing neurons (Cavanaugh et al., 2011) and another which is a cre-dependent GCAMP6 reporter mouse (B6;129S6-*Gt(ROSA)26Sortm96(CAG-GCaMP6s)Hze*/J; Jackson Laboratory) that is currently underway in the Mulligan Lab will result in offspring that express the ultrasensitive genetically encoded Ca^{2+} indicator GCAMP6 in TRPV1 containing neurons negating the need for the use of chemical indicators in the dural skull preparation.

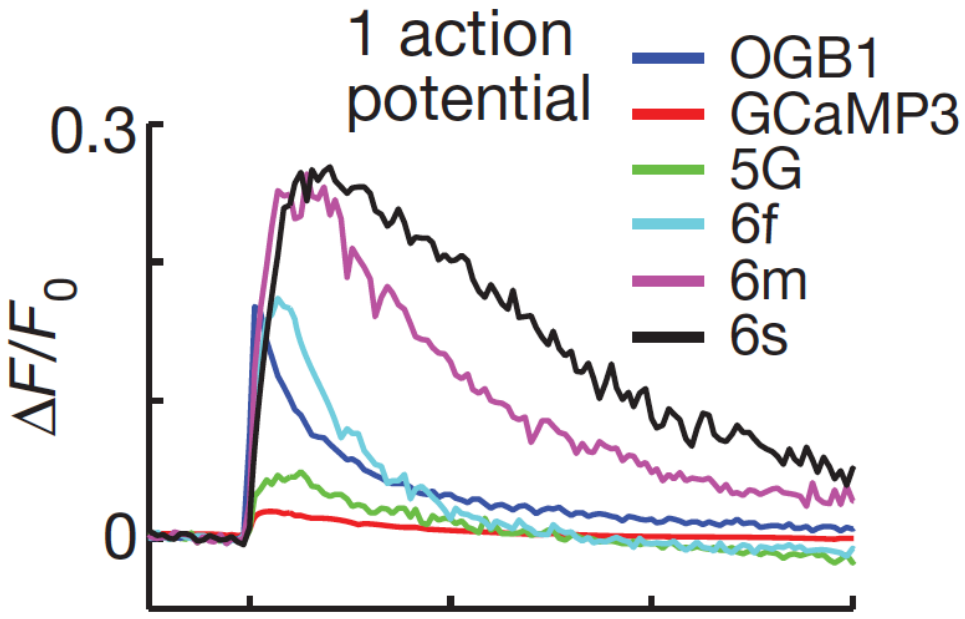

Figure 5.2. Signal- to-noise ratio of the newly developed genetically encoded Ca^{2+} indicator GCAMP6.
The genetically encoded Ca^{2+} indicator GCAMP6 greatly exceeds both the previous version GCAMP3 and one of the most commonly used chemical Ca^{2+} indicators Oregon Green Bapta1 (OGB1). Adapted from (Chen et al., 2013).

One question of the dural skull preparation is whether nociceptive processes such as transduction, propagation and sensitization studied in this preparation can be extrapolated to cutaneous nociceptors. While it has been demonstrated that most properties in TRG cells are similar to those in DRG cells, the chemical responsiveness of some cell types differ slightly between the TRG and DRG (Xu et al., 2010). Also the surrounding tissue in cutaneous and dural nociceptors differs with the peripheral terminals of cutaneous nociceptors residing mainly in the epidermis and dermis (Dubin and Patapoutian, 2010) with the epidermis consisting primarily of keratinocytes and dermis, collagen and elastin fibres (Chong et al., 2013; Lulevich et al., 2010). The cranial nociceptive terminals mainly reside in the dura mater which is primarily made up of fibroblasts and an extracellular matrix consisting of collagen fibrils, microfibrils and elastic fibres (Adeeb et al., 2012). Despite the differences in composition, both tissues appear to be exposed to a similar array of immune cells that are believed to take part in nociceptive processes including mast cells, macrophages, dendritic cells, neutrophils and lymphocytes (Chong et al., 2013; Kipnis et al., 2012; Levy et al., 2007) indicating that nociceptive processes such as neurogenic inflammation and peripheral sensitization may be very similar in both the skin and dura mater.

5.6 Future directions

There are a number of future directions in which the dural skull preparation could go potentially, including the study of specific nociceptive processes, screening of novel analgesic compounds, and finally, with the development of new genetically encoded Ca^{2+} indicators, the dural skull preparation could be extended to *in vivo* studies.

5.6.1 Study of nociceptive processes

Much of our understanding on nociceptive transduction is based on experiments using the isolated cell bodies of nociceptors and it is very possible that differences in the expression of transduction channels exist between the somata and terminals. The dural skull preparation provides the opportunity to test transduction mechanisms identified in the somata, in the peripheral terminals of nociceptors and also allows for the identification of new transduction channels. Although the activation of nociceptors in experiments within this dissertation was restricted to electrical and chemical stimulation, all forms of noxious stimuli including mechanical and thermal stimuli could potentially be used in the dural-skull preparation, which allows for the identification and testing of numerous different types of transduction channels. For example the recently identified mechanical piezo channels (Kim et al., 2012) or two-pore potassium channels (Honore, 2007) which have been implicated in nociception could be tested.

The neuropeptide CGRP is a very potent vasodilator and it is thought that the release of this neuropeptide from nociceptors may contribute to neurogenic inflammation and migraine through vascular mechanisms (Durham, 2008; Kilo et al., 1997). The extent to which CGRP-containing nociceptors need to be activated to cause vasodilation and the exact mechanism through which it occurs could potentially be resolved using the dural skull preparation. Initial data I gathered indicates vasodilation caused by CGRP containing nociceptor activation could be

induced by brief trains of action potentials (20 seconds at 5Hz) in individual CGRP-fibres and this activations results in reliable, repeatable, localized arteriole dilation (Figure 5.3).

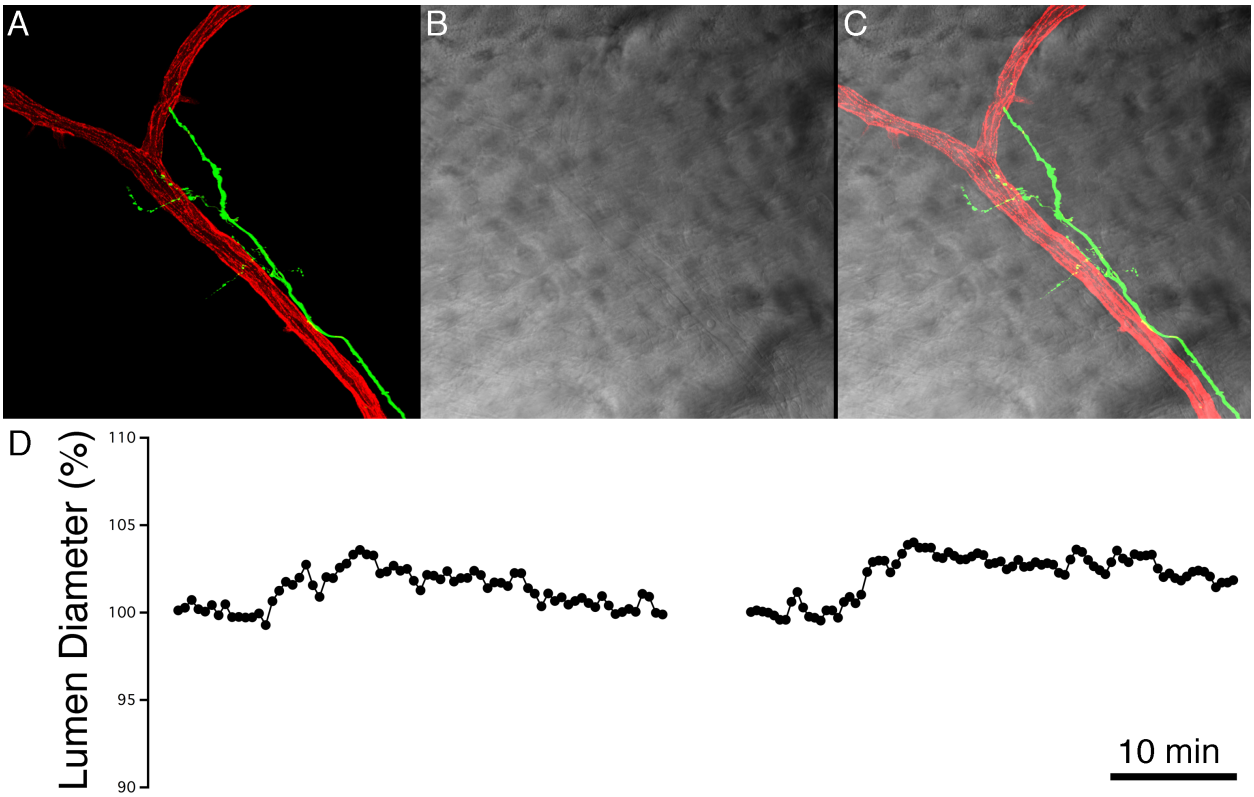

Figure 5.3 CGRP-fibre stimulation causes repeated, localized arteriole dilation.
A) EGFP-CGRP fibre and Alexa-594 IB4 stained (intracardial perfusion) arteriole overlay image shows the close CGRP-fibre-vessel relationship common to these terminating fibres in the meninges (Fricke *et al*. 2001). **B**) IR-transmitted image of the same arteriole shown in (A) and the IR- transmitted and fluorescence image overlay (**C**). **D**) Luminal diameter changes over time from fibre stimulation (20 seconds at 5Hz).

Not only can the process of CGRP-induced vasodilation be observed in the dural-skull preparation, but I have also developed a novel experimental approach to assay the actual release of CGRP from the terminals. CGRP in nociceptors is packaged in large dense core vesicles (Matteoli et al., 1988) and I found that incubation with the acidotropic fluorescent probe DND-99 selectively labels the CGRP large dense core vesicles in a manner similar to LDCVs labeled in chromaffin cells (Duncan et al., 2003). CGRP fibres maintain 1 – 3 LDCVs per terminal bouton (Fricke et al., 1997; Zhang et al., 1995). I have found that brief application of the TRPV1 agonist capsaicin evokes stochastic loss of the DND-99 labeled LDCVs suggesting CGRP release (Figure 5.4) and have also found stochastic loss of labeled LDCVs with photolysis of caged capsaicin and electrical stimulation of the fibre.

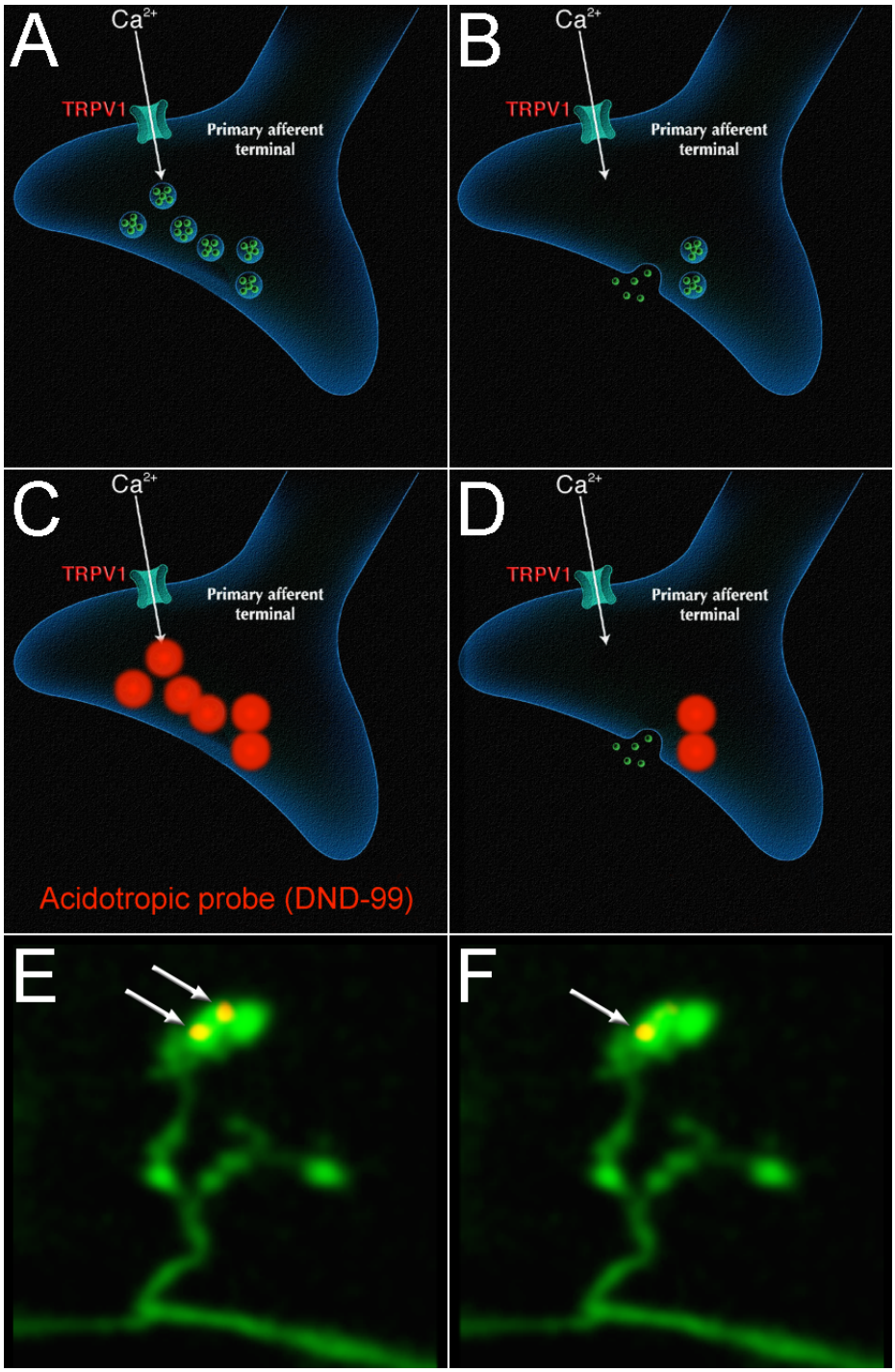

Figure 5.4. TRPV1 channel evoked release of DND-99 labeled CGRP dense core vesicles. (**A – D**) series of schematic figures illustrating a primary afferent terminal in which the TRPV1 channel mediates the release of CGRP dense core vesicles labeled with the acidotropic probe DND-99 (red). **E**) Image of a CGRP-EGFP terminal that shows two dense core vesicles loaded with DND-99 (two arrows). **F**) Brief application of the TRPV1 agonist capsaicin evoked the stochastic loss of one of the DND-99 labeled vesicles suggesting release of a dense core vesicle (single arrow shows remaining vesicle).

Because both nociceptors and a variety of immune cells including macrophages, dendritic cells and mast cells can be easily identified and also labeled with Ca^{2+} indicators in the dural skull preparation, it offers the only opportunity that I am aware of, besides in a culture dish, to directly study immune cell-nociceptor interaction. In the dural skull preparation I have been able to easily identify mast cells with transmitted light or bath application of toluidine blue (Sridharan and Shankar, 2012), as well as to develop a novel method to label macrophages and dendritic cells in live tissue with a bath application of conjugated monoclonal antibodies (Figure 5.5). Since CGRP is considered a key neuropeptide in nociceptor-immune cell interaction (Assas et al., 2014), the dural skull preparation potentially allows for the study of immune cell recruitment, activation and mast cell degranulation through the activation of CGRP containing nociceptors.

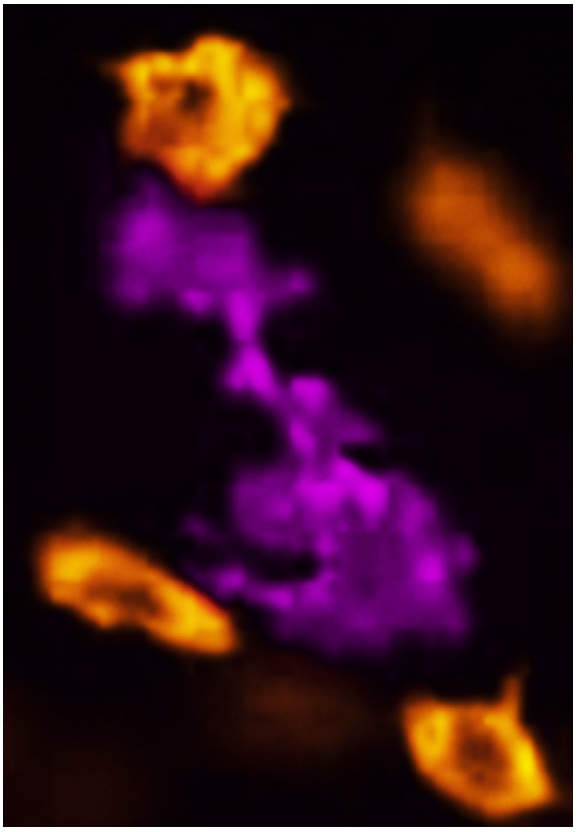

Figure 5.5. Dual macrophage and dendritic cell labeling in live tissue.
Dendritic cells and macrophages were labeled in live tissue with a bath application of fluorescent molecule conjugated monoclonal antibodies. Dendritic cells (purple) were labeled using a rat anti-mouse MHC Class II RT1B Alexa Fluor® 488 monoclonal antibody and macrophages (orange) labeled using a rat anti-mouse CD163 (ED2) Alexa Fluor® 647 antibody.

5.6.2 Testing of novel compounds for analgesia

The ability to study the pain sensing peripheral terminals of nociceptors, in an environment that closely reflects *in vivo* conditions, offers the unique opportunity to identify new analgesics that have been proposed in behavioural models of nociception or somata *in vitro* models. There are a large number of recently developed pain related toxins (Klinger et al., 2012; Rowe et al., 2013; Yang et al., 2013), antibodies (Gearing et al., 2013; Lee et al., 2014) and chemicals that have been tested in *in vitro* somata or behaviourally that could be easily tested in the dural skull preparation to identify their effectiveness in the peripheral terminals of nociceptors.

The dural skull preparation also offers the opportunity to study the mechanisms by which currently used analgesics work in the peripheral terminals and to develop new peripherally restricted analgesics, such as peripherally restricted opioids. I had the opportunity of successfully testing a recently developed peripherally restricted μ-opioid agonist (Furst et al., 2005) in the dural skull preparation (Baillie et al., 2015), that may potentially lead to analgesia without the unwanted side effects (Stein, 2013).

In experiments performed that are not included in this dissertation I had the opportunity to test several selective, small-molecule Na^+ and Ca^{2+} channel blockers being developed for analgesic purpose by the lab of Dr. Terry Snutch at The University of British Columbia. Interestingly differences in the effectiveness of a few of the molecules were found to exist when results in the preparation were compared to previous results gathered in nociceptor somata indicating the utility of the dural-skull preparation in testing novel analgesic compounds.

5.6.3 *In vivo* study of dural nociceptors

It can be inferred by the title of numerous recent reviews (Dodick, 2008; Lambert, 2010; Levy, 2010; Messlinger, 2009) that much of the current understanding regarding the pathophysiological mechanisms of migraine remain speculative in nature and it has also been proposed that the pharmaceutical industry is losing interest in developing new drugs to treat migraine (Olesen and Jansen-Olesen, 2012). Both of these issues have been attributed to a lack of a reliable animal model of the disorder to understand the pathophysiological mechanisms and test novel compounds.

The creation of ultra-sensitive genetically encoded Ca^{2+} indicators as described above offers the ability to extend the dural skull preparation *in vivo* while leaving the cranium completely intact. I have been able to perform *in vivo* imaging of the mouse dura through a thinned-skull optical window, which leaves the skull intact and allows the dural nociceptive fibres, vasculature, and immune cells to remain completely undisturbed. The thinness of the skull at the location of the optical window, together with the proximity of the dural fibres beneath the skull allows for excellent optical resolution to be achieved while imaging through the skull in the anesthetized animal (Figure 5.6).

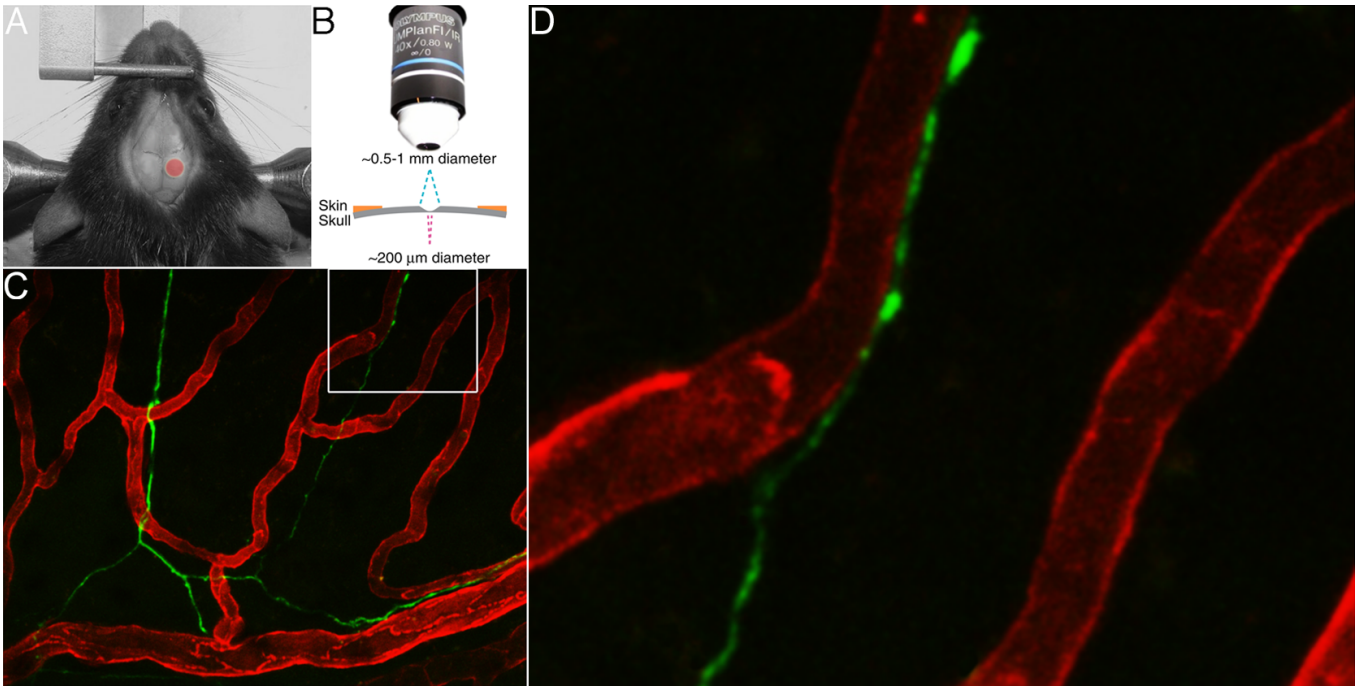

Figure 5.6 Thinned-skull optical window technique for imaging of sensory fibres in live mice.
A) Mouse shown in the head-mounted position (to reduce movement artifacts during imaging). The highlighted circle marks the anatomical location of the thinned-skull optical window. **B**) A schematic diagram showing the imaging lens and cross section of the skull with relative sizes of the thinned imaging area (thinnest region is ~20 µm in thickness and ~200 µm in diameter). **C**) *In vivo* two- photon overlay image showing EGFP-CGRP fibres (green) and Alexa-594 conjugated isolectin B4 stained arterioles (red) (tail vein injection). **D**) A terminating sensory fibre at higher magnification from boxed area in (C).

Two methods can be used to initiate activation of the dural nociceptors and are considered a "migraine model" in mice; the activation of a cortical speading depression (CSD) event (Eikermann-Haerter and Moskowitz, 2008) and the infusion of glyceryl trinitrite (GTN) (Olesen and Jansen-Olesen, 2012).

5.6.3.1 Cortical spreading depression

One key event that may be responsible for the initiation of migraine appears to be cortical spreading depression (CSD), a self-propagating wave of tissue depolarization that migrates without a loss in depolarization amplitude and is followed by a prolonged period of suppressed neural activity (Lauritzen, 1994; Leao, 1944; Olsen, 1995). In up to 30% of migraine sufferers there is a pre-headache experience termed 'aura'; a transient disorder of cerebral cortical functioning that typically manifests as visual disturbances that drift across the visual field. Additional sensory and/or motor changes can also occur that are believed to be caused by CSD (Goadsby, 2007). For the ~70% of migraineurs that do not experience aura, it may be that CSD is in clinically less conspicuous brain regions compared with the visual cortex and may be the trigger of migraine pain ostensibly without a 'perceived' aura (Ayata, 2010).

CSD can be experimentally provoked by topical application of chemicals like KCl, by mechanical stimulation of the cortex with a pin prick or by electrical stimulation of the cortex (Eikermann-Haerter and Moskowitz, 2008). The experimental process for studying activation of dural nociceptors caused by a CSD event involves making a very small hole away from the imaging region and mechanically, chemically or electrically stimulating the cortex to induce CSD followed by monitoring of the Ca^{2+} activity in the nociceptors. The *in vivo* thinned skull method also allows for observation of dural vasculature through a simple tail vein injection of Alexa-594 conjugated isolectin B4 (Figure 5.6).

5.6.3.2 Nitric oxide donors

Nitric oxide (NO) is a versatile gaseous signaling molecule that plays a key role in a variety of biological processes including inflammation and neurotransmission. NO is suggested to be a key constituent in the generation of nociceptive processes in migraine pain (Olesen, 2008; Olesen et al., 1993; Olesen et al., 1995). In migraineurs, the infusion of the NO donors, nitroglycerin and nitroprusside induced headaches that were reported to be very similar to spontaneous migraine attacks (Christiansen et al., 1999). Nitrite, a stable product of NO degradation, has been found in high concentrations in the venous outflow from the head during migraine attacks (Sarchielli et al., 2000) and elevated venous nitrite levels exist even between attacks in migraineurs when compared with healthy controls (D'amico et al., 2002). Similarly, clinical trials suggest that blockade of endogenous NO generation can be successful in alleviating migraine pain (Lassen et al., 1997).

The infusion of nitric oxide donors has been extended to rodents to create an animal model of migraine (Bates et al., 2010; Iversen et al., 1989; Pardutz et al., 2000) and could potentially be used with the thinned skull preparation in transgenic mice expressing GCMP6 in TRPV1 containing neurons to monitor peripheral afferent activity during nitric oxide induced headache. The role the dural vasculature plays in nitric oxide induced headache could also be observed with a tail vein injection of Alexa-594 conjugated isolectin B4.

The development of the dural-skull preparation to study signaling in the peripheral terminals of nociceptors and the ability to extend the study of peripheral terminals *in vivo* will hopefully help unravel some of the poorly understood mechanisms of both peripheral nociceptive signaling and migraine headache.

REFERENCES

Abdel Samad, O., Liu, Y., Yang, F.C., Kramer, I., Arber, S., and Ma, Q. (2010). Characterization of two Runx1-dependent nociceptor differentiation programs necessary for inflammatory versus neuropathic pain. Molecular pain *6*, 45.

Adeeb, N., Mortazavi, M.M., Tubbs, R.S., and Cohen-Gadol, A.A. (2012). The cranial dura mater: a review of its history, embryology, and anatomy. Child's nervous system : ChNS : official journal of the International Society for Pediatric Neurosurgery *28*, 827-837.

Ahn, A.H., and Basbaum, A.I. (2005). Where do triptans act in the treatment of migraine? Pain *115*, 1-4.

Akerman, S., Holland, P.R., and Goadsby, P.J. (2011). Diencephalic and brainstem mechanisms in migraine. Nat Rev Neurosci *12*, 570-584.

Akerman, S., Holland, P.R., Lasalandra, M.P., and Goadsby, P.J. (2010). Inhibition of trigeminovascular dural nociceptive afferents by Ca(2+)-activated K(+) (MaxiK/BK(Ca)) channel opening. Pain *151*, 128-136.

Akins, P.T., and McCleskey, E.W. (1993). Characterization of potassium currents in adult rat sensory neurons and modulation by opioids and cyclic AMP. Neuroscience *56*, 759-769.

Al-Aqaba, M.A., Fares, U., Suleman, H., Lowe, J., and Dua, H.S. (2010). Architecture and distribution of human corneal nerves. The British journal of ophthalmology *94*, 784-789.

Al-Khrasani, M., Spetea, M., Friedmann, T., Riba, P., Kiraly, K., Schmidhammer, H., and Furst, S. (2007). DAMGO and 6beta-glycine substituted 14-O-methyloxymorphone but not morphine show peripheral, preemptive antinociception after systemic administration in a mouse visceral pain model and high intrinsic efficacy in the isolated rat vas deferens. Brain research bulletin *74*, 369-375.

Altier, C., Dale, C.S., Kisilevsky, A.E., Chapman, K., Castiglioni, A.J., Matthews, E.A., Evans, R.M., Dickenson, A.H., Lipscombe, D., Vergnolle, N.*, et al.* (2007). Differential role of N-type calcium channel splice isoforms in pain. The Journal of neuroscience : the official journal of the Society for Neuroscience *27*, 6363-6373.

Altier, C., and Zamponi, G.W. (2004). Targeting Ca2+ channels to treat pain: T-type versus N-type. Trends Pharmacol Sci *25*, 465-470.

Amatrudo, J.M., Olson, J.P., Lur, G., Chiu, C.Q., Higley, M.J., and Ellis-Davies, G.C. (2014). Wavelength-selective one- and two-photon uncaging of GABA. ACS chemical neuroscience *5*, 64-70.

Amrutkar, D.V., Ploug, K.B., Olesen, J., and Jansen-Olesen, I. (2011). Role for Voltage Gated Calcium Channels in Calcitonin Gene-Related Peptide Release in the Rat Trigeminovascular System. Neuroscience *172*, 510-517.

Ansel, J.C., Brown, J.R., Payan, D.G., and Brown, M.A. (1993). Substance P selectively activates TNF-alpha gene expression in murine mast cells. Journal of immunology *150*, 4478-4485.

Arvieu, L., Mauborgne, A., Bourgoin, S., Oliver, C., Feltz, P., Hamon, M., and Cesselin, F. (1996). Sumatriptan inhibits the release of CGRP and substance P from the rat spinal cord. Neuroreport *7*, 1973-1976.

Assas, B.M., Pennock, J.I., and Miyan, J.A. (2014). Calcitonin gene-related peptide is a key neurotransmitter in the neuro-immune axis. Frontiers in neuroscience *8*, 23.

Ayata, C. (2010). Cortical spreading depression triggers migraine attack: pro. Headache *50*, 725-730.

Babos, M.B., Grady, B., Wisnoff, W., and McGhee, C. (2013). Pathophysiology of pain. Disease-a-month : DM *59*, 330-358.

Baillie, L.D., Ahn, A.H., and Mulligan, S.J. (2012). Sumatriptan inhibition of N-type calcium channel mediated signaling in dural CGRP terminal fibres. Neuropharmacology *63*, 362-367.

Baillie, L.D., Hagen, V., Gardner, K.M., and Mulligan, S.J. (2011). Functional imaging within individual pain fibres ex vivo with optical microscopy. Journal of neuroscience methods *198*, 274-279.

Baillie, L.D., Schmidhammer, H., and Mulligan, S.J. (2015). Peripheral mu-opioid receptor mediated inhibition of calcium signaling and action potential-evoked calcium fluorescent transients in primary afferent CGRP nociceptive terminals. Neuropharmacology *93*, 267-273.

Bartsch, T., Knight, Y.E., and Goadsby, P.J. (2004). Activation of 5-HT(1B/1D) receptor in the periaqueductal gray inhibits nociception. Ann Neurol *56*, 371-381.

Basbaum, A.I., Bautista, D.M., Scherrer, G., and Julius, D. (2009). Cellular and molecular mechanisms of pain. Cell *139*, 267-284.

Bates, E.A., Nikai, T., Brennan, K.C., Fu, Y.H., Charles, A.C., Basbaum, A.I., Ptacek, L.J., and Ahn, A.H. (2010). Sumatriptan alleviates nitroglycerin-induced mechanical and thermal allodynia in mice. Cephalalgia : an international journal of headache *30*, 170-178.

Benarroch, E.E. (2011). CGRP: sensory neuropeptide with multiple neurologic implications. Neurology *77*, 281-287.

Benemei, S., Nicoletti, P., Capone, J.G., and Geppetti, P. (2009). CGRP receptors in the control of pain and inflammation. Current opinion in pharmacology *9*, 9-14.

Bennett, G.J., and Xie, Y.K. (1988). A Peripheral Mononeuropathy in Rat That Produces Disorders of Pain Sensation Like Those Seen in Man. Pain *33*, 87-107.

Berkefeld, H., Fakler, B., and Schulte, U. (2010). Ca2+-activated K+ channels: from protein complexes to function. Physiological reviews *90*, 1437-1459.

Bigal, M.E., Krymchantowski, A.V., and Ho, T. (2009). Migraine in the triptan era: progresses achieved, lessons learned and future developments. Arq Neuropsiquiatr *67*, 559-569.

Bileviciute-Ljungar, I., Spetea, M., Guo, Y., Schutz, J., Windisch, P., and Schmidhammer, H. (2006). Peripherally mediated antinociception of the mu-opioid receptor agonist 2-[(4,5alpha-epoxy-3-hydroxy-14beta-methoxy-17-methylmorphinan-6beta-yl)amino]ace tic acid (HS-731) after subcutaneous and oral administration in rats with carrageenan-induced hindpaw inflammation. The Journal of pharmacology and experimental therapeutics *317*, 220-227.

Bird, G.C., Han, J.S., Fu, Y., Adwanikar, H., Willis, W.D., and Neugebauer, V. (2006). Pain-related synaptic plasticity in spinal dorsal horn neurons: role of CGRP. Molecular pain *2*, 31.

Blanchard, J.W., Eade, K.T., Szucs, A., Lo Sardo, V., Tsunemoto, R.K., Williams, D., Sanna, P.P., and Baldwin, K.K. (2015). Selective conversion of fibroblasts into peripheral sensory neurons. Nature neuroscience *18*, 25-+.

Bollmann, J.H., and Sakmann, B. (2005). Control of synaptic strength and timing by the release-site Ca2+ signal. Nature neuroscience *8*, 426-434.

Boyce-Rustay, J.M., Honore, P., and Jarvis, M.F. (2010). Animal models of acute and chronic inflammatory and nociceptive pain. Methods in molecular biology *617*, 41-55.

Brain, S.D., and Grant, A.D. (2004). Vascular actions of calcitonin gene-related peptide and adrenomedullin. Physiological reviews *84*, 903-934.

Brain, S.D., Williams, T.J., Tippins, J.R., Morris, H.R., and MacIntyre, I. (1985). Calcitonin gene-related peptide is a potent vasodilator. Nature *313*, 54-56.

Brower, V. (2000). New paths to pain relief. Nature biotechnology *18*, 387-391.

Bruce, A.N. (1913). Vaso-dilator axon reflexes. Q J Exp Physiol *6*, 339 - 354.

Burgos-Vega, C., Moy, J., and Dussor, G. (2015). Meningeal afferent signaling and the pathophysiology of migraine. Progress in molecular biology and translational science *131*, 537-564.

Burkey, T.H., Hingtgen, C.M., and Vasko, M.R. (2004). Isolation and culture of sensory neurons from the dorsal-root ganglia of embryonic or adult rats. Methods in molecular medicine *99*, 189-202.

Buzas, B., and Cox, B.M. (1997). Quantitative analysis of MU and delta opioid receptor gene expression in rat brain and peripheral ganglia using competitive polymerase chain reaction. Neuroscience *76*, 479-489.

Buzzi, M.G., Carter, W.B., Shimizu, T., Heath, H., 3rd, and Moskowitz, M.A. (1991). Dihydroergotamine and sumatriptan attenuate levels of CGRP in plasma in rat superior sagittal sinus during electrical stimulation of the trigeminal ganglion. Neuropharmacology *30*, 1193-1200.

Carr, R.W., and Brock, J.A. (2002). Electrophysiology of corneal cold receptor nerve terminals. Advances in experimental medicine and biology *508*, 19-23.

Carr, R.W., Pianova, S., and Brock, J.A. (2002). The effects of polarizing current on nerve terminal impulses recorded from polymodal and cold receptors in the guinea-pig cornea. The Journal of general physiology *120*, 395-405.

Carr, R.W., Pianova, S., Fernandez, J., Fallon, J.B., Belmonte, C., and Brock, J.A. (2003). Effects of heating and cooling on nerve terminal impulses recorded from cold-sensitive receptors in the guinea-pig cornea. The Journal of general physiology *121*, 427-439.

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D., and Julius, D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature *389*, 816-824.

Cavanaugh, D.J., Chesler, A.T., Jackson, A.C., Sigal, Y.M., Yamanaka, H., Grant, R., O'Donnell, D., Nicoll, R.A., Shah, N.M., Julius, D*., et al.* (2011). Trpv1 reporter mice reveal highly restricted brain distribution and functional expression in arteriolar smooth muscle cells. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 5067-5077.

Cesare, P., and McNaughton, P. (1996). A novel heat-activated current in nociceptive neurons and its sensitization by bradykinin. Proceedings of the National Academy of Sciences of the United States of America *93*, 15435-15439.

Chambers, S.M., Qi, Y.C., Mica, Y., Lee, G., Zhang, X.J., Niu, L., Bilsland, J., Cao, L.S., Stevens, E., Whiting, P*., et al.* (2012). Combined small-molecule inhibition accelerates developmental timing and converts human pluripotent stem cells into nociceptors. Nat Biotechnol *30*, 715-+.

Chen, J.J., Dymshitz, J., and Vasko, M.R. (1997). Regulation of opioid receptors in rat sensory neurons in culture. Mol Pharmacol *51*, 666-673.

Chen, S.R., Cai, Y.Q., and Pan, H.L. (2009). Plasticity and emerging role of BKCa channels in nociceptive control in neuropathic pain. Journal of neurochemistry *110*, 352-362.

Chen, T.W., Wardill, T.J., Sun, Y., Pulver, S.R., Renninger, S.L., Baohan, A., Schreiter, E.R., Kerr, R.A., Orger, M.B., Jayaraman, V*., et al.* (2013). Ultrasensitive fluorescent proteins for imaging neuronal activity. Nature *499*, 295-300.

Chiu, I.M., von Hehn, C.A., and Woolf, C.J. (2012). Neurogenic inflammation and the peripheral nervous system in host defense and immunopathology. Nature neuroscience *15*, 1063-1067.

Chong, S.Z., Evrard, M., and Ng, L.G. (2013). Lights, camera, and action: vertebrate skin sets the stage for immune cell interaction with arthropod-vectored pathogens. Frontiers in immunology *4*, 286.

Christiansen, I., Thomsen, L.L., Daugaard, D., Ulrich, V., and Olesen, J. (1999). Glyceryl trinitrate induces attacks of migraine without aura in sufferers of migraine with aura. Cephalalgia : an international journal of headache *19*, 660-667.

Chung, M.K., Guler, A.D., and Caterina, M.J. (2008). TRPV1 shows dynamic ionic selectivity during agonist stimulation. Nature neuroscience *11*, 555-564.

Clapham, D.E. (2003). TRP channels as cellular sensors. Nature *426*, 517-524.

Cohen, S.P., and Mao, J. (2014). Neuropathic pain: mechanisms and their clinical implications. Bmj *348*, f7656.

Cook, A.J., Woolf, C.J., Wall, P.D., and McMahon, S.B. (1987). Dynamic receptive field plasticity in rat spinal cord dorsal horn following C-primary afferent input. Nature *325*, 151-153.

Cox, J.J., Reimann, F., Nicholas, A.K., Thornton, G., Roberts, E., Springell, K., Karbani, G., Jafri, H., Mannan, J., Raashid, Y*., et al.* (2006). An SCN9A channelopathy causes congenital inability to experience pain. Nature *444*, 894-898.

Cumberbatch, M.J., Hill, R.G., and Hargreaves, R.J. (1997). Rizatriptan has central antinociceptive effects against durally evoked responses. European journal of pharmacology *328*, 37-40.

Cunha, T.M., Verri, W.A., Jr., Vivancos, G.G., Moreira, I.F., Reis, S., Parada, C.A., Cunha, F.Q., and Ferreira, S.H. (2004). An electronic pressure-meter nociception paw test for mice. Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica [et al] *37*, 401-407.

Currie, K.P. (2010). G protein modulation of CaV2 voltage-gated calcium channels. Channels (Austin) *4*, 497-509.

D'amico, D., Ferraris, A., Leone, M., Catania, A., Carlin, A., Grazzi, L., and Bussone, G. (2002). Increased plasma nitrites in migraine and cluster headache patients in interictal period: basal hyperactivity of L-arginine-NO pathway? Cephalalgia : an international journal of headache *22*, 33-36.

D'amour, F.E., and Smith, D.L. (1941). A method for determining loss of pain sensation. Journal of Pharmacology and Experimental Therapeutics *72*, 74-79.

Dib-Hajj, S.D., Yang, Y., Black, J.A., and Waxman, S.G. (2013). The Na(V)1.7 sodium channel: from molecule to man. Nature reviews Neuroscience *14*, 49-62.

Ding, W., Stohl, L.L., Wagner, J.A., and Granstein, R.D. (2008). Calcitonin gene-related peptide biases Langerhans cells toward Th2-type immunity. Journal of immunology *181*, 6020-6026.

Dodge, F.A., Jr., and Rahamimoff, R. (1967). Co-operative action a calcium ions in transmitter release at the neuromuscular junction. The Journal of physiology *193*, 419-432.

Dodick, D., and Silberstein, S. (2006). Central sensitization theory of migraine: clinical implications. Headache *46 Suppl 4*, S182-191.

Dodick, D.W. (2008). Examining the essence of migraine--is it the blood vessel or the brain? A debate. Headache *48*, 661-667.

Dorland, W.A.N. (2003). Dorland's Illustrated Medical Dictionary, 30th Edition, 30th edn (Philadelphia Saunders/Elsevier).

Dubin, A.E., and Patapoutian, A. (2010). Nociceptors: the sensors of the pain pathway. The Journal of clinical investigation *120*, 3760-3772.

Duncan, R.R., Greaves, J., Wiegand, U.K., Matskevich, I., Bodammer, G., Apps, D.K., Shipston, M.J., and Chow, R.H. (2003). Functional and spatial segregation of secretory vesicle pools according to vesicle age. Nature *422*, 176-180.

Durham, P.L. (2006). Calcitonin gene-related peptide (CGRP) and migraine. Headache *46*, S3-S8.

Durham, P.L. (2008). Inhibition of calcitonin gene-related peptide function: a promising strategy for treating migraine. Headache *48*, 1269-1275.

Durham, P.L., and Russo, A.F. (1999). Regulation of calcitonin gene-related peptide secretion by a serotonergic antimigraine drug. The Journal of neuroscience : the official journal of the Society for Neuroscience *19*, 3423-3429.

Durham, P.L., and Russo, A.F. (2002). New insights into the molecular actions of serotonergic antimigraine drugs. Pharmacol Ther *94*, 77-92.

Durham, P.L., and Russo, A.F. (2003). Stimulation of the calcitonin gene-related peptide enhancer by mitogen-activated protein kinases and repression by an antimigraine drug in trigeminal ganglia neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience *23*, 807-815.

Durham, P.L., and Vause, C.V. (2010). Calcitonin gene-related peptide (CGRP) receptor antagonists in the treatment of migraine. CNS drugs *24*, 539-548.

Ebersberger, A. (2001). Physiology of meningeal innervation: aspects and consequences of chemosensitivity of meningeal nociceptors. Microscopy research and technique *53*, 138-146.

Ebersberger, A., Charbel Issa, P., Vanegas, H., and Schaible, H.G. (2000). Differential effects of calcitonin gene-related peptide and calcitonin gene-related peptide 8-37 upon responses to N-methyl-D-aspartate or (R, S)-alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate in spinal nociceptive neurons with knee joint input in the rat. Neuroscience *99*, 171-178.

Edvinsson, L., and Ho, T.W. (2010). CGRP receptor antagonism and migraine. Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics *7*, 164-175.

Eftekhari, S., and Edvinsson, L. (2010). Possible sites of action of the new calcitonin gene-related peptide receptor antagonists. Therapeutic advances in neurological disorders *3*, 369-378.

Eftekhari, S., Salvatore, C.A., Calamari, A., Kane, S.A., Tajti, J., and Edvinsson, L. (2010). Differential distribution of calcitonin gene-related peptide and its receptor components in the human trigeminal ganglion. Neuroscience *169*, 683-696.

Eikermann-Haerter, K., and Moskowitz, M.A. (2008). Animal models of migraine headache and aura. Current opinion in neurology *21*, 294-300.

Endres-Becker, J., Heppenstall, P.A., Mousa, S.A., Labuz, D., Oksche, A., Schafer, M., Stein, C., and Zollner, C. (2007). Mu-opioid receptor activation modulates transient receptor potential vanilloid 1 (TRPV1) currents in sensory neurons in a model of inflammatory pain. Mol Pharmacol *71*, 12-18.

Esteban, J.A., Shi, S.H., Wilson, C., Nuriya, M., Huganir, R.L., and Malinow, R. (2003). PKA phosphorylation of AMPA receptor subunits controls synaptic trafficking underlying plasticity. Nature neuroscience *6*, 136-143.

Fakler, B., and Adelman, J.P. (2008). Control of K(Ca) channels by calcium nano/microdomains. Neuron *59*, 873-881.

Farazifard, R., Safarpour, F., Sheibani, V., and Javan, M. (2005). Eye-wiping test: a sensitive animal model for acute trigeminal pain studies. Brain research Brain research protocols *16*, 44-49.

Feindel, W., Penfield, W., and Mc, N.F. (1960). The tentorial nerves and Iocalization of intracranial pain in man. Neurology *10*, 555-563.

Fertleman, C.R., Baker, M.D., Parker, K.A., Moffatt, S., Elmslie, F.V., Abrahamsen, B., Ostman, J., Klugbauer, N., Wood, J.N., Gardiner, R.M*., et al.* (2006). SCN9A mutations in paroxysmal extreme pain disorder: allelic variants underlie distinct channel defects and phenotypes. Neuron *52*, 767-774.

Fields, H.L., Emson, P.C., Leigh, B.K., Gilbert, R.F., and Iversen, L.L. (1980). Multiple opiate receptor sites on primary afferent fibres. Nature *284*, 351-353.

Fischer, M.J. (2010). Calcitonin gene-related peptide receptor antagonists for migraine. Expert opinion on investigational drugs *19*, 815-823.

Foreman, J.C. (1987). Peptides and Neurogenic Inflammation. Brit Med Bull *43*, 386-400.

Franck, M.C., Stenqvist, A., Li, L., Hao, J., Usoskin, D., Xu, X., Wiesenfeld-Hallin, Z., and Ernfors, P. (2011). Essential role of Ret for defining non-peptidergic nociceptor phenotypes and functions in the adult mouse. The European journal of neuroscience *33*, 1385-1400.

Fricke, B., Andres, K.H., and Von During, M. (2001). Nerve fibers innervating the cranial and spinal meninges: morphology of nerve fiber terminals and their structural integration. Microscopy research and technique *53*, 96-105.

Fricke, B., von During, M., and Andres, K.H. (1997). Topography and immunocytochemical characterization of nerve fibers in the leptomeningeal compartments of the rat. A light- and electron-microscopical study. Cell and tissue research *287*, 11-22.

Fruhstorfer, H., Gross, W., and Selbmann, O. (2001). von Frey hairs: new materials for a new design. European journal of pain *5*, 341-342.

Furst, S., Riba, P., Friedmann, T., Timar, J., Al-Khrasani, M., Obara, I., Makuch, W., Spetea, M., Schutz, J., Przewlocki, R.*, et al.* (2005). Peripheral versus central antinociceptive actions of 6-amino acid-substituted derivatives of 14-O-methyloxymorphone in acute and inflammatory pain in the rat. The Journal of pharmacology and experimental therapeutics *312*, 609-618.

Gamse, R., and Saria, A. (1986). Nociceptive behavior after intrathecal injections of substance P, neurokinin A and calcitonin gene-related peptide in mice. Neuroscience letters *70*, 143-147.

Gearing, D.P., Virtue, E.R., Gearing, R.P., and Drew, A.C. (2013). A fully caninised anti-NGF monoclonal antibody for pain relief in dogs. BMC veterinary research *9*, 226.

Gilbert, D., Funk, K., Dekowski, B., Lechler, R., Keller, S., Mohrlen, F., Frings, S., and Hagen, V. (2007). Caged capsaicins: New tools for the examination of TRPV1 channels in somatosensory neurons. Chembiochem *8*, 89-97.

Goadsby, P.J. (2007). Recent advances in understanding migraine mechanisms, molecules and therapeutics. Trends in molecular medicine *13*, 39-44.

Goadsby, P.J., and Edvinsson, L. (1993). The trigeminovascular system and migraine: studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. Ann Neurol *33*, 48-56.

Goadsby, P.J., Edvinsson, L., and Ekman, R. (1990). Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. Annals of neurology *28*, 183-187.

Goadsby, P.J., Lipton, R.B., and Ferrari, M.D. (2002). Migraine--current understanding and treatment. The New England journal of medicine *346*, 257-270.

Gold, M.S., and Gebhart, G.F. (2010). Nociceptor sensitization in pain pathogenesis. Nature medicine *16*, 1248-1257.

Gover, T.D., Kao, J.P., and Weinreich, D. (2003). Calcium signaling in single peripheral sensory nerve terminals. The Journal of neuroscience : the official journal of the Society for Neuroscience *23*, 4793-4797.

Gover, T.D., Moreira, T.H., Kao, J.P., and Weinreich, D. (2007). Calcium regulation in individual peripheral sensory nerve terminals of the rat. The Journal of physiology *578*, 481-490.

Grace, P.M., Hutchinson, M.R., Maier, S.F., and Watkins, L.R. (2014). Pathological pain and the neuroimmune interface. Nature reviews Immunology *14*, 217-231.

Grandl, J., Kim, S.E., Uzzell, V., Bursulaya, B., Petrus, M., Bandell, M., and Patapoutian, A. (2010). Temperature-induced opening of TRPV1 ion channel is stabilized by the pore domain. Nature neuroscience *13*, 708-714.

Handwerker, H.O. (2010). Classification of nociceptors - To what purpose? Pain *148*, 355-356.

Hargreaves, K., Dubner, R., Brown, F., Flores, C., and Joris, J. (1988). A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain *32*, 77-88.

Hargreaves, R. (2007). New migraine and pain research. Headache *47 Suppl 1*, S26-43.

Harriott, A.M., and Gold, M.S. (2008). Serotonin type 1D receptors (5HTR) are differentially distributed in nerve fibres innervating craniofacial tissues. Cephalalgia : an international journal of headache *28*, 933-944.

Harrison, R.G. (1907). Observations on the living developing nerve fiber. P Soc Exp Biol Med *4*, 140-143.

Heinricher, M.M., Tavares, I., Leith, J.L., and Lumb, B.M. (2009). Descending control of nociception: Specificity, recruitment and plasticity. Brain research reviews *60*, 214-225.

Ho, T.W., Edvinsson, L., and Goadsby, P.J. (2010). CGRP and its receptors provide new insights into migraine pathophysiology. Nat Rev Neurol *6*, 573-582.

Hoffmann, J., and Goadsby, P.J. (2011). New Agents for Acute Treatment of Migraine: CGRP Receptor Antagonists, iNOS Inhibitors. Current treatment options in neurology.

Honore, E. (2007). The neuronal background K2P channels: focus on TREK1. Nature reviews Neuroscience *8*, 251-261.

Honore, P., Wismer, C.T., Mikusa, J., Zhu, C.Z., Zhong, C., Gauvin, D.M., Gomtsyan, A., El Kouhen, R., Lee, C.H., Marsh, K.*, et al.* (2005). A-425619 [1-isoquinolin-5-yl-3-(4-trifluoromethyl-benzyl)-urea], a novel transient receptor potential type V1 receptor antagonist, relieves pathophysiological pain associated with inflammation and tissue injury in rats. The Journal of pharmacology and experimental therapeutics *314*, 410-421.

Hoskin, K.L., and Goadsby, P.J. (1998). Comparison of more and less lipophilic serotonin (5HT(1B/1D)) agonists in a model of trigeminovascular nociception in cat. Exp Neurol *150*, 45-51.

Huang, J., Han, C., Estacion, M., Vasylyev, D., Hoeijmakers, J.G., Gerrits, M.M., Tyrrell, L., Lauria, G., Faber, C.G., Dib-Hajj, S.D.*, et al.* (2014). Gain-of-function mutations in sodium channel Na(v)1.9 in painful neuropathy. Brain : a journal of neurology *137*, 1627-1642.

Humphrey, P.P., and Feniuk, W. (1991). Mode of action of the anti-migraine drug sumatriptan. Trends Pharmacol Sci *12*, 444-446.

Humphrey, P.P.A., and Goadsby, P.J. (1994). The Mode of Action of Sumatriptan Is Vascular - a Debate. Cephalalgia : an international journal of headache *14*, 401-410.

Iftinca, M.C., and Zamponi, G.W. (2009). Regulation of neuronal T-type calcium channels. Trends Pharmacol Sci *30*, 32-40.

Iversen, H.K., Olesen, J., and Tfelthansen, P. (1989). Intravenous Nitroglycerin as an Experimental-Model of Vascular Headache - Basic Characteristics. Pain *38*, 17-24.

Iwaszkiewicz, K.S., Schneider, J.J., and Hua, S. (2013). Targeting peripheral opioid receptors to promote analgesic and anti-inflammatory actions. Frontiers in pharmacology *4*, 132.

Jancsó, G.b. (2009). Neurogenic inflammation in health and disease, 1st edn (Amsterdam: Elsevier).

Jansen-Olesen, I., Mortensen, A., and Edvinsson, L. (1996). Calcitonin gene-related peptide is released from capsaicin-sensitive nerve fibres and induces vasodilatation of human cerebral arteries concomitant with activation of adenylyl cyclase. Cephalalgia : an international journal of headache *16*, 310-316.

Janssen, P.A., Niemegeers, C.J., and Dony, J.G. (1963). The inhibitory effect of fentanyl and other morphine-like analgesics on the warm water induced tail withdrawl reflex in rats. Arzneimittel-Forschung *13*, 502-507.

Jevtovic-Todorovic, V., and Todorovic, S.M. (2006). The role of peripheral T-type calcium channels in pain transmission. Cell calcium *40*, 197-203.

Ji, R.R., Kohno, T., Moore, K.A., and Woolf, C.J. (2003). Central sensitization and LTP: do pain and memory share similar mechanisms? Trends in neurosciences *26*, 696-705.

Jordt, S.E., and Julius, D. (2002). Molecular basis for species-specific sensitivity to "hot" chili peppers. Cell *108*, 421-430.

Jordt, S.E., Tominaga, M., and Julius, D. (2000). Acid potentiation of the capsaicin receptor determined by a key extracellular site. Proceedings of the National Academy of Sciences of the United States of America *97*, 8134-8139.

Julius, D., and Basbaum, A.I. (2001). Molecular mechanisms of nociception. Nature *413*, 203-210.

Kandel, E.R. (2013). Principles of neural science, 5th edn (New York: McGraw-Hill).

Kangrga, I., Larew, J.S., and Randic, M. (1990). The effects of substance P and calcitonin gene-related peptide on the efflux of endogenous glutamate and aspartate from the rat spinal dorsal horn in vitro. Neuroscience letters *108*, 155-160.

Kangrga, I., and Randic, M. (1990). Tachykinins and calcitonin gene-related peptide enhance release of endogenous glutamate and aspartate from the rat spinal dorsal horn slice. The Journal of neuroscience : the official journal of the Society for Neuroscience *10*, 2026-2038.

Katz, B., and Miledi, R. (1965). The Effect of Calcium on Acetylcholine Release from Motor Nerve Terminals. Proc R Soc Lond B Biol Sci *161*, 496-503.

Kaube, H., Hoskin, K.L., and Goadsby, P.J. (1993). Inhibition by sumatriptan of central trigeminal neurones only after blood-brain barrier disruption. British journal of pharmacology *109*, 788-792.

Kieffer, B.L., and Gaveriaux-Ruff, C. (2002). Exploring the opioid system by gene knockout. Progress in neurobiology *66*, 285-306.

Kilo, S., Harding-Rose, C., Hargreaves, K.M., and Flores, C.M. (1997). Peripheral CGRP release as a marker for neurogenic inflammation: a model system for the study of neuropeptide secretion in rat paw skin. Pain *73*, 201-207.

Kim, S.E., Coste, B., Chadha, A., Cook, B., and Patapoutian, A. (2012). The role of Drosophila Piezo in mechanical nociception. Nature *483*, 209-212.

Kipnis, J., Gadani, S., and Derecki, N.C. (2012). Pro-cognitive properties of T cells. Nature reviews Immunology *12*, 663-669.

Klinger, A.B., Eberhardt, M., Link, A.S., Namer, B., Kutsche, L.K., Schuy, E.T., Sittl, R., Hoffmann, T., Alzheimer, C., Huth, T.*, et al.* (2012). Sea-anemone toxin ATX-II elicits A-fiber-dependent pain and enhances resurgent and persistent sodium currents in large sensory neurons. Molecular pain *8*, 69.

Kostyuk, E.P., and Kostyuk, P.G. (2009). Peculiarities of Ion Channels and Modulation of Their Functions in Neurons Belonging to the Nociceptive System. Neurophysiology *41*, 241-250.

Kuner, R. (2010). Central mechanisms of pathological pain. Nature medicine *16*, 1258-1266.

Lambert, G.A. (2010). The lack of peripheral pathology in migraine headache. Headache *50*, 895-908.

Lassen, L.H., Ashina, M., Christiansen, I., Ulrich, V., and Olesen, J. (1997). Nitric oxide synthase inhibition in migraine. Lancet *349*, 401-402.

Lassen, L.H., Haderslev, P.A., Jacobsen, V.B., Iversen, H.K., Sperling, B., and Olesen, J. (2002). CGRP may play a causative role in migraine. Cephalalgia : an international journal of headache *22*, 54-61.

Latremoliere, A., and Woolf, C.J. (2009). Central sensitization: a generator of pain hypersensitivity by central neural plasticity. The journal of pain : official journal of the American Pain Society *10*, 895-926.

Lauritzen, M. (1994). Pathophysiology of the migraine aura. The spreading depression theory. Brain : a journal of neurology *117 (Pt 1)*, 199-210.

Le Bars, D., Gozariu, M., and Cadden, S.W. (2001). Animal models of nociception. Pharmacological reviews *53*, 597-652.

Le Greves, P., Nyberg, F., Terenius, L., and Hokfelt, T. (1985). Calcitonin gene-related peptide is a potent inhibitor of substance P degradation. European journal of pharmacology *115*, 309-311.

Leao, A.A.P. (1944). Spreading depression of activity in the cerebral cortex. Journal of neurophysiology *7*, 359-390.

Lee, J.H., Park, C.K., Chen, G., Han, Q., Xie, R.G., Liu, T., Ji, R.R., and Lee, S.Y. (2014). A monoclonal antibody that targets a NaV1.7 channel voltage sensor for pain and itch relief. Cell *157*, 1393-1404.

Leiser, S.C., and Moxon, K.A. (2007). Responses of trigeminal ganglion neurons during natural whisking behaviors in the awake rat. Neuron *53*, 117-133.

Lennerz, J.K., Ruhle, V., Ceppa, E.P., Neuhuber, W.L., Bunnett, N.W., Grady, E.F., and Messlinger, K. (2008). Calcitonin receptor-like receptor (CLR), receptor activity-modifying protein 1 (RAMP1), and calcitonin gene-related peptide (CGRP) immunoreactivity in the rat trigeminovascular system: differences between peripheral and central CGRP receptor distribution. The Journal of comparative neurology *507*, 1277-1299.

Levy, D. (2010). Migraine pain and nociceptor activation--where do we stand? Headache *50*, 909-916.

Levy, D., Burstein, R., Kainz, V., Jakubowski, M., and Strassman, A.M. (2007). Mast cell degranulation activates a pain pathway underlying migraine headache. Pain *130*, 166-176.

Levy, D., Jakubowski, M., and Burstein, R. (2004). Disruption of communication between peripheral and central trigeminovascular neurons mediates the antimigraine action of 5HT 1B/1D receptor agonists. Proc Natl Acad Sci U S A *101*, 4274-4279.

Limmroth, V., Katsarava, Z., Liedert, B., Guehring, H., Schmitz, K., Diener, H.C., and Michel, M.C. (2001). An in vivo rat model to study calcitonin gene related peptide release following activation of the trigeminal vascular system. Pain *92*, 101-106.

Limmroth, V., May, A., Auerbach, P., Wosnitza, G., Eppe, T., and Diener, H.C. (1996). Changes in cerebral blood flow velocity after treatment with sumatriptan or placebo and implications for the pathophysiology of migraine. Journal of the neurological sciences *138*, 60-65.

Longmore, J., Shaw, D., Smith, D., Hopkins, R., McAllister, G., Pickard, J.D., Sirinathsinghji, D.J., Butler, A.J., and Hill, R.G. (1997). Differential distribution of 5HT1D- and 5HT1B-immunoreactivity within the human trigemino-cerebrovascular system: implications for the discovery of new antimigraine drugs. Cephalalgia : an international journal of headache *17*, 833-842.

Lu, R., Lukowski, R., Sausbier, M., Zhang, D.D., Sisignano, M., Schuh, C.D., Kuner, R., Ruth, P., Geisslinger, G., and Schmidtko, A. (2014). BKCa channels expressed in sensory neurons modulate inflammatory pain in mice. Pain *155*, 556-565.

Lulevich, V., Yang, H.Y., Isseroff, R.R., and Liu, G.Y. (2010). Single cell mechanics of keratinocyte cells. Ultramicroscopy *110*, 1435-1442.

Lundberg, J.M., Franco-Cereceda, A., Hua, X., Hokfelt, T., and Fischer, J.A. (1985). Co-existence of substance P and calcitonin gene-related peptide-like immunoreactivities in sensory nerves in relation to cardiovascular and bronchoconstrictor effects of capsaicin. European journal of pharmacology *108*, 315-319.

Madrid, R., Donovan-Rodriguez, T., Meseguer, V., Acosta, M.C., Belmonte, C., and Viana, F. (2006). Contribution of TRPM8 channels to cold transduction in primary sensory neurons and peripheral nerve terminals. The Journal of neuroscience : the official journal of the Society for Neuroscience *26*, 12512-12525.

Marmigere, F., and Ernfors, P. (2007). Specification and connectivity of neuronal subtypes in the sensory lineage. Nature reviews Neuroscience *8*, 114-127.

Matteoli, M., Haimann, C., Torritarelli, F., Polak, J.M., Ceccarelli, B., and Decamilli, P. (1988). Differential Effect of Alpha-Latrotoxin on Exocytosis from Small Synaptic Vesicles and from Large Dense-Core Vesicles Containing Calcitonin Gene-Related Peptide at the Frog Neuromuscular-Junction. Proceedings of the National Academy of Sciences of the United States of America *85*, 7366-7370.

McCormack, D.G., Mak, J.C., Coupe, M.O., and Barnes, P.J. (1989). Calcitonin gene-related peptide vasodilation of human pulmonary vessels. Journal of applied physiology *67*, 1265-1270.

McCoy, E.S., Taylor-Blake, B., Street, S.E., Pribisko, A.L., Zheng, J., and Zylka, M.J. (2013). Peptidergic CGRPalpha primary sensory neurons encode heat and itch and tonically suppress sensitivity to cold. Neuron *78*, 138-151.

Mehrotra, S., Gupta, S., Chan, K.Y., Villalon, C.M., Centurion, D., Saxena, P.R., and MaassenVanDenBrink, A. (2008). Current and prospective pharmacological targets in relation to antimigraine action. Naunyn Schmiedebergs Arch Pharmacol *378*, 371-394.

Melli, G., and Hoke, A. (2009). Dorsal Root Ganglia Sensory Neuronal Cultures: a tool for drug discovery for peripheral neuropathies. Expert opinion on drug discovery *4*, 1035-1045.

Messlinger, K. (2009). Migraine: where and how does the pain originate? Experimental brain research *196*, 179-193.

Messlinger, K., Hanesch, U., Baumgartel, M., Trost, B., and Schmidt, R.F. (1993). Innervation of the dura mater encephali of cat and rat: ultrastructure and calcitonin gene-related peptide-like and substance P-like immunoreactivity. Anatomy and embryology *188*, 219-237.

Meyer, R.A., Ringkamp, R., Campbell, J.N., and and Raja, S.N. (2005). Peripheral Mechanisms of Cutaneous Nociception. In Wall and Melzack's Texbook of Pain, M.K.S. McMahon, ed. (Elsevier), pp. 3-34.

Moskowitz, M.A., and Cutrer, F.M. (1993). SUMATRIPTAN: a receptor-targeted treatment for migraine. Annual review of medicine *44*, 145-154.

Muller, L.J., Marfurt, C.F., Kruse, F., and Tervo, T.M. (2003). Corneal nerves: structure, contents and function. Experimental eye research *76*, 521-542.

Natura, G., von Banchet, G.S., and Schaible, H.G. (2005). Calcitonin gene-related peptide enhances TTX-resistant sodium currents in cultured dorsal root ganglion neurons from adult rats. Pain *116*, 194-204.

Niederkorn, J.Y. (2005). Corneal immune privilege. The ocular surface *3*, S158-160.

Nockemann, D., Rouault, M., Labuz, D., Hublitz, P., McKnelly, K., Reis, F.C., Stein, C., and Heppenstall, P.A. (2013). The K+ channel GIRK2 is both necessary and sufficient for peripheral opioid-mediated analgesia. Embo Mol Med *5*, 1263-1277.

Oh, U. (2006). The nociceptive membrane (Amsterdam ; Boston: Elsevier).

Oh, U., Hwang, S.W., and Kim, D. (1996). Capsaicin activates a nonselective cation channel in cultured neonatal rat dorsal root ganglion neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience *16*, 1659-1667.

Olesen, J. (2008). The role of nitric oxide (NO) in migraine, tension-type headache and cluster headache. Pharmacology & therapeutics *120*, 157-171.

Olesen, J., Burstein, R., Ashina, M., and Tfelt-Hansen, P. (2009). Origin of pain in migraine: evidence for peripheral sensitisation. The Lancet Neurology *8*, 679-690.

Olesen, J., Iversen, H.K., and Thomsen, L.L. (1993). Nitric oxide supersensitivity: a possible molecular mechanism of migraine pain. Neuroreport *4*, 1027-1030.

Olesen, J., and Jansen-Olesen, I. (2012). Towards a reliable animal model of migraine. Cephalalgia : an international journal of headache *32*, 578-580.

Olesen, J., Thomsen, L.L., Lassen, L.H., and Olesen, I.J. (1995). The nitric oxide hypothesis of migraine and other vascular headaches. Cephalalgia : an international journal of headache *15*, 94-100.

Olsen, T.S. (1995). Pathophysiology of the migraine aura: the spreading depression theory. Brain : a journal of neurology *118 (Pt 1)*, 307-308.

Pardutz, A., Krizbai, I., Multon, S., Vecsei, L., and Schoenen, J. (2000). Systemic nitroglycerin increases nNOS levels in rat trigeminal nucleus caudalis. Neuroreport *11*, 3071-3075.

Pettit, D.L., Wang, S.S., Gee, K.R., and Augustine, G.J. (1997). Chemical two-photon uncaging: a novel approach to mapping glutamate receptors. Neuron *19*, 465-471.

Pietrobon, D., and Striessnig, J. (2003). Neurobiology of migraine. Nature reviews Neuroscience *4*, 386-398.

Potrebic, S., Ahn, A.H., Skinner, K., Fields, H.L., and Basbaum, A.I. (2003). Peptidergic nociceptors of both trigeminal and dorsal root ganglia express serotonin 1D receptors: Implications for the selective antimigraine action of triptans. Journal of Neuroscience *23*, 10988-10997.

Randall, L.O., and Selitto, J.J. (1957). A method for measurement of analgesic activity on inflamed tissue. Archives internationales de pharmacodynamie et de therapie *111*, 409-419.

Razzaque, Z., Heald, M.A., Pickard, J.D., Maskell, L., Beer, M.S., Hill, R.G., and Longmore, J. (1999). Vasoconstriction in human isolated middle meningeal arteries: determining the contribution of 5-HT1B- and 5-HT1F-receptor activation. British journal of clinical pharmacology *47*, 75-82.

Recober, A., Kuburas, A., Zhang, Z., Wemmie, J.A., Anderson, M.G., and Russo, A.F. (2009). Role of calcitonin gene-related peptide in light-aversive behavior: implications for migraine. The Journal of neuroscience : the official journal of the Society for Neuroscience *29*, 8798-8804.

Reeh, P.W. (1986). Sensory receptors in mammalian skin in an in vitro preparation. Neuroscience letters *66*, 141-146.

Reichling, D.B., and Levine, J.D. (1997). Heat transduction in rat sensory neurons by calcium-dependent activation of a cation channel. Proceedings of the National Academy of Sciences of the United States of America *94*, 7006-7011.

Renganathan, M., Cummins, T.R., and Waxman, S.G. (2001). Contribution of Na(v)1.8 sodium channels to action potential electrogenesis in DRG neurons. Journal of neurophysiology *86*, 629-640.

Richardson, J.D., and Vasko, M.R. (2002). Cellular mechanisms of neurogenic inflammation. The Journal of pharmacology and experimental therapeutics *302*, 839-845.

Rochlitzer, S., Veres, T.Z., Kuhne, K., Prenzler, F., Pilzner, C., Knothe, S., Winkler, C., Lauenstein, H.D., Willart, M., Hammad, H.*, et al.* (2011). The neuropeptide calcitonin gene-related peptide affects allergic airway inflammation by modulating dendritic cell function. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology *41*, 1609-1621.

Rowe, A.H., Xiao, Y., Rowe, M.P., Cummins, T.R., and Zakon, H.H. (2013). Voltage-gated sodium channel in grasshopper mice defends against bark scorpion toxin. Science *342*, 441-446.

Sah, P. (1996). Ca(2+)-activated K+ currents in neurones: types, physiological roles and modulation. Trends in neurosciences *19*, 150-154.

Sarchielli, P., Alberti, A., Codini, M., Floridi, A., and Gallai, V. (2000). Nitric oxide metabolites, prostaglandins and trigeminal vasoactive peptides in internal jugular vein blood during spontaneous migraine attacks. Cephalalgia : an international journal of headache *20*, 907-918.

Sarchielli, P., Pini, L.A., Zanchin, G., Alberti, A., Maggioni, F., Rossi, C., Floridi, A., and Calabresi, P. (2006). Clinical-biochemical correlates of migraine attacks in rizatriptan responders and non-responders. Cephalalgia : an international journal of headache *26*, 257-265.

Saria, A. (1984). Substance P in sensory nerve fibres contributes to the development of oedema in the rat hind paw after thermal injury. British journal of pharmacology *82*, 217-222.

Schaible, H.G. (2007). Peripheral and central mechanisms of pain generation. Handbook of experimental pharmacology, 3-28.

Schneggenburger, R., and Neher, E. (2005). Presynaptic calcium and control of vesicle fusion. Current opinion in neurobiology *15*, 266-274.

Scott, A., Khan, K.M., Cook, J.L., and Duronio, V. (2004). What is "inflammation"? Are we ready to move beyond Celsus? British journal of sports medicine *38*, 248-249.

Sehgal, N., Smith, H.S., and Manchikanti, L. (2011). Peripherally acting opioids and clinical implications for pain control. Pain physician *14*, 249-258.

Seybold, V.S. (2009). The role of peptides in central sensitization. Handbook of experimental pharmacology, 451-491.

Sidach, S.S., and Mintz, I.M. (2000). Low-affinity blockade of neuronal N-type Ca channels by the spider toxin omega-agatoxin-IVA. J Neurosci *20*, 7174-7182.

Sleight, A.J., Cervenka, A., and Peroutka, S.J. (1990). In vivo effects of sumatriptan (GR 43175) on extracellular levels of 5-HT in the guinea pig. Neuropharmacology *29*, 511-513.

Snider, W.D., and McMahon, S.B. (1998). Tackling Pain at the Source- New Ideas about Nociceptors. Neuron *20*, 629-632.

Snutch, T.P. (2005). Targeting chronic and neuropathic pain: the N-type calcium channel comes of age. NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics *2*, 662-670.

Soyguder, Z. (1999). Lectin and neuropeptide labeling in whole-mount preparation of meninges in the rat. Turk J Vet Anim Sci *23*, 495-498.

Spetea, M., Friedmann, T., Riba, P., Schutz, J., Wunder, G., Langer, T., Schmidhammer, H., and Furst, S. (2004). In vitro opioid activity profiles of 6-amino acid substituted derivatives of 14-O-methyloxymorphone. European journal of pharmacology *483*, 301-308.

Sprenger, T., and Goadsby, P.J. (2009). Migraine pathogenesis and state of pharmacological treatment options. BMC Med *7*, 71.

Sridharan, G., and Shankar, A.A. (2012). Toluidine blue: A review of its chemistry and clinical utility. Journal of oral and maxillofacial pathology : JOMFP *16*, 251-255.

Stein, C. (1993). Peripheral mechanisms of opioid analgesia. Anesthesia and analgesia *76*, 182-191.

Stein, C. (1995). The control of pain in peripheral tissue by opioids. The New England journal of medicine *332*, 1685-1690.

Stein, C. (2013). Targeting pain and inflammation by peripherally acting opioids. Frontiers in pharmacology *4*, 123.

Stein, C., Hassan, A.H., Przewlocki, R., Gramsch, C., Peter, K., and Herz, A. (1990). Opioids from immunocytes interact with receptors on sensory nerves to inhibit nociception in inflammation. Proceedings of the National Academy of Sciences of the United States of America *87*, 5935-5939.

Stein, C., and Kuchler, S. (2012). Non-Analgesic Effects of Opioids: Peripheral Opioid Effects on Inflammation and Wound Healing. Curr Pharm Design *18*, 6053-6069.

Stein, C., and Machelska, H. (2011). Modulation of peripheral sensory neurons by the immune system: implications for pain therapy. Pharmacological reviews *63*, 860-881.

Stein, C., Pfluger, M., Yassouridis, A., Hoelzl, J., Lehrberger, K., Welte, C., and Hassan, A.H.S. (1996). No tolerance to peripheral morphine analgesia in presence of opioid expression in inflamed synovia. Journal of Clinical Investigation *98*, 793-799.

Stein, C., Schafer, M., and Machelska, H. (2003). Attacking pain at its source: new perspectives on opioids. Nature medicine *9*, 1003-1008.

Stein, C., and Zollner, C. (2009). Opioids and sensory nerves. Handbook of experimental pharmacology, 495-518.

Stepien, A., Jagustyn, P., Trafny, E.A., and Widerkiewicz, K. (2003). Suppressing effect of the serotonin 5HT1B/D receptor agonist rizatriptan on calcitonin gene-related peptide (CGRP) concentration in migraine attacks. Neurologia i neurochirurgia polska *37*, 1013-1023.

Strassman, A.M., and Levy, D. (2006). Response properties of dural nociceptors in relation to headache. Journal of neurophysiology *95*, 1298-1306.

Strassman, A.M., Weissner, W., Williams, M., Ali, S., and Levy, D. (2004). Axon diameters and intradural trajectories of the dural innervation in the rat. The Journal of comparative neurology *473*, 364-376.

Sun, R.Q., Tu, Y.J., Lawand, N.B., Yan, J.Y., Lin, Q., and Willis, W.D. (2004). Calcitonin gene-related peptide receptor activation produces PKA- and PKC-dependent mechanical hyperalgesia and central sensitization. Journal of neurophysiology *92*, 2859-2866.

Szallasi, A., Cortright, D.N., Blum, C.A., and Eid, S.R. (2007). The vanilloid receptor TRPV1: 10 years from channel cloning to antagonist proof-of-concept. Nat Rev Drug Discov *6*, 357-372.

Takayama, Y., Uta, D., Furue, H., and Tominaga, M. (2015). Pain-enhancing mechanism through interaction between TRPV1 and anoctamin 1 in sensory neurons. Proceedings of the National Academy of Sciences of the United States of America.

Takazawa, T., and MacDermott, A.B. (2010). Synaptic pathways and inhibitory gates in the spinal cord dorsal horn. Annals of the New York Academy of Sciences *1198*, 153-158.

Tfelt-Hansen, P., De Vries, P., and Saxena, P.R. (2000). Triptans in migraine: a comparative review of pharmacology, pharmacokinetics and efficacy. Drugs *60*, 1259-1287.

Tfelt-Hansen, P.C. (2010). Does sumatriptan cross the blood-brain barrier in animals and man? J Headache Pain *11*, 5-12.

Tfelt-Hansen, P.C., and Koehler, P.J. (2011). One hundred years of migraine research: major clinical and scientific observations from 1910 to 2010. Headache *51*, 752-778.

Todorovic, S.M., and Jevtovic-Todorovic, V. (2006). The role of T-type calcium channels in peripheral and central pain processing. CNS & neurological disorders drug targets *5*, 639-653.

Todorovic, S.M., and Jevtovic-Todorovic, V. (2007). Regulation of T-type calcium channels in the peripheral pain pathway. Channels (Austin) *1*, 238-245.

Todorovic, S.M., and Jevtovic-Todorovic, V. (2011). T-type voltage-gated calcium channels as targets for the development of novel pain therapies. British journal of pharmacology *163*, 484-495.

Tsantoulas, C., and McMahon, S.B. (2014). Opening paths to novel analgesics: the role of potassium channels in chronic pain. Trends in neurosciences *37*, 146-158.

Tvedskov, J.F., Lipka, K., Ashina, M., Iversen, H.K., Schifter, S., and Olesen, J. (2005). No increase of calcitonin gene-related peptide in jugular blood during migraine. Ann Neurol *58*, 561-568.

Uddman, R., Edvinsson, L., Ekblad, E., Hakanson, R., and Sundler, F. (1986). Calcitonin gene-related peptide (CGRP): perivascular distribution and vasodilatory effects. Regulatory peptides *15*, 1-23.

Venkatachalam, K., and Montell, C. (2007). TRP channels. Annual review of biochemistry *76*, 387-417.

Villalon, C.M., and Olesen, J. (2009). The role of CGRP in the pathophysiology of migraine and efficacy of CGRP receptor antagonists as acute antimigraine drugs. Pharmacology & therapeutics *124*, 309-323.

Wainger, B.J., Buttermore, E.D., Oliveira, J.T., Mellin, C., Lee, S., Saber, W.A., Wang, A.J., Ichida, J.K., Chiu, I.M., Barrett, L.*, et al.* (2015). Modeling pain in vitro using nociceptor neurons reprogrammed from fibroblasts. Nature neuroscience *18*, 17-+.

Wang, H., and Woolf, C.J. (2005). Pain TRPs. Neuron *46*, 9-12.

Waxman, S.G., and Zamponi, G.W. (2014). Regulating excitability of peripheral afferents: emerging ion channel targets. Nature neuroscience *17*, 153-163.

Weiss, N. (2009). Regulation of N-type calcium channels by G-proteins: multiple pathways to control calcium entry into neurons. Channels (Austin) *3*, 219-220.

Woolf, C.J., and Ma, Q. (2007). Nociceptors--noxious stimulus detectors. Neuron *55*, 353-364.

Woolfe, G., and Macdonald, A.D. (1944). The evaluation of the analgesic action of Pethidine hydrochloride (Demerol). Journal of Pharmacology and Experimental Therapeutics *80*, 300-307.

Xiao, Y., Richter, J.A., and Hurley, J.H. (2008). Release of glutamate and CGRP from trigeminal ganglion neurons: Role of calcium channels and 5-HT1 receptor signaling. Molecular pain *4*, 12.

Xie, W.R. (2011). Assessment of Pain in Animals. Neuromethods *49*, 1-+.

Xu, S., Ono, K., and Inenaga, K. (2010). Electrophysiological and chemical properties in subclassified acutely dissociated cells of rat trigeminal ganglion by current signatures. Journal of neurophysiology *104*, 3451-3461.

Yang, S., Xiao, Y., Kang, D., Liu, J., Li, Y., Undheim, E.A., Klint, J.K., Rong, M., Lai, R., and King, G.F. (2013). Discovery of a selective NaV1.7 inhibitor from centipede venom with analgesic efficacy exceeding morphine in rodent pain models. Proceedings of the National Academy of Sciences of the United States of America *110*, 17534-17539.

Young, W.S., Wamsley, J.K., Zarbin, M.A., and Kuhar, M.J. (1980). Opioid Receptors Undergo Axonal Flow. Science *210*, 76-78.

Yusaf, S.P., Goodman, J., Pinnock, R.D., Dixon, A.K., and Lee, K. (2001). Expression of voltage-gated calcium channel subunits in rat dorsal root ganglion neurons. Neuroscience letters *311*, 137-141.

Zamponi, G.W., Lewis, R.J., Todorovic, S.M., Arneric, S.P., and Snutch, T.P. (2009). Role of voltage-gated calcium channels in ascending pain pathways. Brain research reviews *60*, 84-89.

Zhang, X., Bean, A.J., Wiesenfeld-Hallin, Z., Xu, X.J., and Hokfelt, T. (1995). Ultrastructural studies on peptides in the dorsal horn of the rat spinal cord--III. Effects of peripheral axotomy with special reference to galanin. Neuroscience *64*, 893-915.

Zhang, X.F., Gopalakrishnan, M., and Shieh, C.C. (2003). Modulation of action potential firing by iberiotoxin and NS1619 in rat dorsal root ganglion neurons. Neuroscience *122*, 1003-1011.

Zimmermann, K., Hein, A., Hager, U., Kaczmarek, J.S., Turnquist, B.P., Clapham, D.E., and Reeh, P.W. (2009). Phenotyping sensory nerve endings in vitro in the mouse. Nature protocols *4*, 174-196.

Zucker, R.S. (1993). Calcium and transmitter release. Journal of physiology, Paris *87*, 25-36.